UREOLYTIC $CaCO_3$ PRECIPITATION FOR IMMOBILIZATION OF ARSENIC IN AN AQUIFER SYSTEM

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By

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ABSTRACT

The objective of this study was to precipitate $CaCO_3$ in a groundwater media to reduce dissolved arsenic concentrations. In this study a mixture of ureolytic calcite and aragonite were precipitated using groundwater as the media. Although precipitation of carbonate was successful using Ardkenneth groundwater, arsenic concentrations were not reduced. Ureolytic calcite and aragonite precipitated using broth as the media and resulted in a decrease in arsenic concentrations of up to 88% from the initial 0.7 $\mu g\ L^{-1}$ concentration. Ureolytic carbonate precipitation required the inoculation of ureolytic cultures isolated from groundwater into both the groundwater and broth media. Precipitates in the inoculation experiments were identified using infrared spectroscopy techniques.

The decrease in arsenic concentrations in the inoculated urea treated broth samples compared to the groundwater samples was attributed to the greater amounts of precipitate formed in the broth media. The broth had a free Ca(II) concentration of 1300 $mg\ L^{-1}$ whereas the Ardkenneth groundwater had a free Ca(II) concentration of 36 to 42 $mg\ L^{-1}$. The higher free Ca(II) concentrations in the broth media would account for the higher yield of carbonate precipitate, making Ca(II) concentration a limiting factor in ureolytic $CaCO_3$ remediation techniques.

Formation of a visible precipitate required the addition of nitrate to the broth and groundwater samples. The inoculated cultures, being denitrifiers, required a nitrate source. Ca(II) ion concentrations decreased in the different media without the addition of nitrate, but no visible precipitate formed.

Laboratory experiments using Ardkenneth groundwater and treatments of 0.03 *M* urea did not decrease the Ca(II) ion concentrations or reduce arsenic in solution. These results suggest that inoculation with selected ureolytic cultures was needed to optimize the precipitation of $CaCO_3$ in a natural groundwater system.

The results of this study suggest that arsenic was reduced by the precipitation of ureolytic $CaCO_3$. Arsenic reduced by ureolytic $CaCO_3$ precipitation required adequate levels of Ca(II) ions, higher than those found in the Ardkenneth aquifer. Successful precipitation of $CaCO_3$ by ureolytic organisms also required an adequate cell density. Thus, inoculation with ureolytic cultures optimized the broth and groundwater media for $CaCO_3$ precipitation.

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LIST OF ABBREVIATIONS

ΔG	Gibbs free energy
AFS	Atomic fluorescence spectroscopy
ARD	Acid rock drainage
ATP	Adenosine tri-phosphate
C_T	Total carbon
CFU	Colony forming unit
DIC	Dissolved inorganic carbon
DMA	Dimethylarsine
DOC	Dissolved organic carbon
EMF	Electron motive force
FTIR	Fourier transform infrared
FTIR-PAS	Fourier transform infrared photoacustic spectroscopy
IAP	Ion activity product
ICP-AES	Inductively coupled plasma atomic emission spectrometer
ICP-MS	Inductively coupled plasma mass spectrometer
IR	Infrared
ISA	Ionic strength adjuster
ISE	Ion selective electrode
LSD	Least significant difference
NHE	Normal hydrogen electrode
OC	Organic carbon
PVC	Polyvinyl chloride
SAM	*S*-adenosylmethyltransferase
SI	Saturation index
TOC	Total organic carbon
WHO	World health organization
XRD	X-ray diffraction

1.0 INTRODUCTION

Arsenic is a natural toxic element of concern found in the environment. Arsenic is part of group 15 of the periodic table and is classified as a metalloid. Concentrations of arsenic vary within the earth's crust and are approximately 1.5 mg kg^{-1} (Plant et al., 2004; Smedley and Kinniburgh, 2002) Arsenic is a chalcophile under the Goldschmidt classification of the elements, meaning it is often associated with sulfur and forms several minerals including realgar (AsS), orpiment (As_2S_3) and arsenopyrite (FeAsS) to name a few (Goldschmidt, 1954; Plant et al., 2004).

Arsenic exists as both inorganic and organic forms in several oxidation states -3, 0, +3, and +5 (Goldschmidt, 1954; Plant et al., 2004). Arsenic found in groundwater is predominately dissolved arsenate [As(V)] and arsenite [As(III)]. The geochemistry of arsenic and interactions with other minerals such as oxides and sulphides control the mobility of arsenic in a groundwater system (Stollenwerk, 2003). Arsenic often sorbs to oxide mineral surfaces forming inner-sphere complexes (Plant et al., 2004; Stollenwerk, 2003). Arsenic sorption and desorption strongly depend on pH and Eh. Increasing Eh, or oxidation of a groundwater system can lead to arsenic contamination from sulphide minerals (Rodriguez et al., 2004). The resulting sulphide oxidation lowers the pH, thus allowing the arsenic to remain in solution (Rodriguez et al., 2004).

Groundwater contaminated by arsenic is a well known global issue affecting millions of people in Bangladesh alone. The source of arsenic in the Bengal delta is not a

result of sulphide oxidation, but instead the microbial reduction of iron oxides (Ahmed et al., 2004; Akai et al., 2004; Anawar et al., 2002; Anawar et al., 2003). Arsenic concentrations in Bangladesh range from 30 to 750 μg L^{-1}; well over the World Health Organization's limits of 10 μg L^{-1} (Anawar et al., 2003). People in this region experience a multitude of health problems associated with the elevated arsenic levels in the groundwater. Health problems associated with arsenic poisoning include arsenic-induced dermatoses, skin cancer, as well as cancer in the lungs, bladder, and liver (Roy and Saha, 2002).

In the Ardkenneth aquifer of southern Saskatchewan, Canada, arsenic is limited to the semi permeable overlying aquitard. Within the aquifer itself the arsenic levels range from 0.2 to 0.7 μg L^{-1}, within the guidelines for safe drinking. The source of arsenic is possibly oxidation of sulphides as elevated levels of arsenic occur in the oxic zone of the overlying Snakebite Member (Yan et al., 2000). Within the first 15 m of the Snakebite Member the concentrations of both As(III) and As(V) are elevated, and then increase once again at depth within a redox transition zone (Yan et al., 2000). The elevated concentrations of arsenic within the oxidized zone are of concern as increasing demands for fresh water within Canada and around the globe place more pressure on aquifers such as the Ardkenneth because current water usage removes water faster that the local recharge rates. Although arsenic sorption to iron oxides is preferred, other more stable minerals, such as carbonates, are thought to also be a beneficial remediation strategy (Stollenwerk, 2003).

Chemical precipitation of $CaCO_3$ typically occurs in freshwater systems low in Mg(II) or as a secondary mineral deposited from hydrothermal waters (Geyssant, 2001; Stumm and Morgan, 1995). $CaCO_3$ precipitation occurs when there is over saturation of

calcium, inorganic carbon, and an abundance of dissolved CO_2 (Stumm and Morgan, 1995). In a closed system, oversaturation is reached at lower carbonate concentrations than in an open system because of limited CO_2 (Stumm and Morgan, 1995).

Bacteria can change the regional geochemical conditions to favour reactions such as $CaCO_3$ precipitation (Fujita et al., 2000). In closed systems, with limited dissolved inorganic carbon (DIC), bacteria can alter the system by producing DIC through their metabolic pathways (Hammes, 2003). When urea is added to the system bacteria will hydrolyze the urea, producing ammonium and increasing pH. This increase in pH will produce HCO_3^-, an important form of DIC for $CaCO_3$ precipitation. As well as increasing the necessary DIC for $CaCO_3$ formation, bacteria also act as crystal nucleation sites because their cell walls possess a net negative charge (Fujita et al., 2004; Hammes, 2003).

The hypothesis for this study was that bacterial $CaCO_3$ precipitation could immobilize arsenic in groundwater. This study aimed to precipitate biogenic $CaCO_3$ using urea hydrolysis and natural groundwater as the media to reduce dissolved arsenic concentrations. Also, this study aimed to identify the bacteria responsible for $CaCO_3$ precipitation isolated from the Ardkenneth groundwater.

Groundwater was collected from piezometer 4A, installed by the Saskatchewan Research Council (SRC), near Riverhurst, Saskatchewan. Bioreactors were constructed to investigate if the addition of urea to the indigenous groundwater microbial community would (1) precipitate $CaCO_3$ and (2) reduce arsenic concentrations in the groundwater. In addition, ureolytic microorganisms were enriched and isolated from the groundwater and used inoculants for broth and groundwater experiments.

This thesis is presented in five chapters; following this introduction (Chapter 1) is the literature review offered in Chapter 2. Chapter 3 discusses the bioreactor experiments with indigenous microorganisms and all groundwater analysis methods. In Chapter 4 the results from the inoculation experiments are presented and discussed. Chapter 5 is a general conclusion and summarizes the laboratory work performed in this study. Results of both experimental types are discussed to form a general conclusion and recommendations for future works in arsenic bioremediation techniques.

2.0 LITERATURE REVIEW

2.1 Arsenic Sources and Chemistry

2.1.1 Arsenic geology: minerals and sources

Arsenic is a natural element found in the earth's crust, and ranks 47th in abundance of the 88 elements within the earth's crust (Plant et al., 2004). Arsenic is found in group 15 of the periodic table and is classified as a chalcophilic metalloid. Arsenic has one stable isotope ^{75}As and exists in several oxidation states. Arsenite [As(III)] can substitute for iron [Fe(III)] and aluminum [Al(III)] and thus can be found as an impurity in a variety of minerals such as pyrite (FeS_2), chalcopyrite ($CuFeS_2$), or pyrrhotite ($Fe_{x-1}S_x$) and some silicate minerals (Ehrlich, 2002; Plant et al., 2004). Also, arsenic and selenium can substitute for each other which is relevant to arsenic toxicity as small amounts of Se are essential for both human and animal health (Plant et al., 2004) .

Arsenic is brought to the earth's crust by various igneous processes such as volcanism or intrusions beneath the surface. The crustal abundance of arsenic is around 1.5 mg kg^{-1} (Plant et al., 2004; Smedley and Kinniburgh, 2002). Because arsenic is a chalcophile 60% of arsenic minerals are in the form of arsenates, 20% are sulphides, and the final 20% are arsenides, arsenites, oxides, and various polymorphs of elemental arsenic (Plant et al., 2004; Smedley and Kinniburgh, 2002). Some examples of natural arsenic minerals include; realgar [AsS], pararealgar [AsS] which results from ultraviolet alteration of realgar, alacranite [AsS], orpiment [As_2S_3], Arsenopyrite [FeAsS] (Fig.

2.1), or sulfosalts such as enargite [Cu_3AsS], and can be associated with other elements such as Se or tellurium (Te) to name a few (Burns and Percival, 2001; Ehrlich, 2002; King, 2002). Arsenic incorporation in primary rock-forming minerals is limited as As(III) can substitute for Fe(III) or Al(III). Igneous and metamorphic rocks have an average arsenic concentration of 1 to 10 mg kg^{-1}, with similar concentrations found in carbonate minerals (Plant et al., 2004). Arsenic concentrations in sedimentary rocks are more variable as arsenic is highly dependant on the geochemical content of the source lithologies (Table 2.1). The highest sedimentary arsenic concentrations of 20 to 200 mg kg^{-1} typically are found in organic and/or sulphide rich shales, sedimentary ironstones, phosphatic rocks and some coals (Plant et al., 2004; Smedley and Kinniburgh, 2002). Arsenic is concentrated in the clay or other fine grained components of most sedimentary rocks, especially if they are rich in sulphide minerals, secondary iron oxides, organic matter, and phosphates. Glacial till, alluvial sands, and lake sediments have much lower average arsenic concentrations (<1 to 15 mg kg^{-1}) (Plant et al., 2004; Webb, 1978). Soil arsenic concentrations typically are similar to that of sediments and dependant on the parent material's original arsenic concentrations (Table 2.1).

The primary source of arsenic in groundwater is from groundwater aquifer sediment interactions (Freeze and Cherry, 1979). The West Bengal Delta is a well known area of high arsenic contamination with arsenic levels ranging from 30 to 750 μg L^{-1} (Anawar et al., 2003). The arsenic contamination in the Bengal Delta is a result of microbial reduction or breakdown of Fe oxides. The natural peat deposits in the region provide indigenous bacteria with a carbon source, allowing for the reduction of

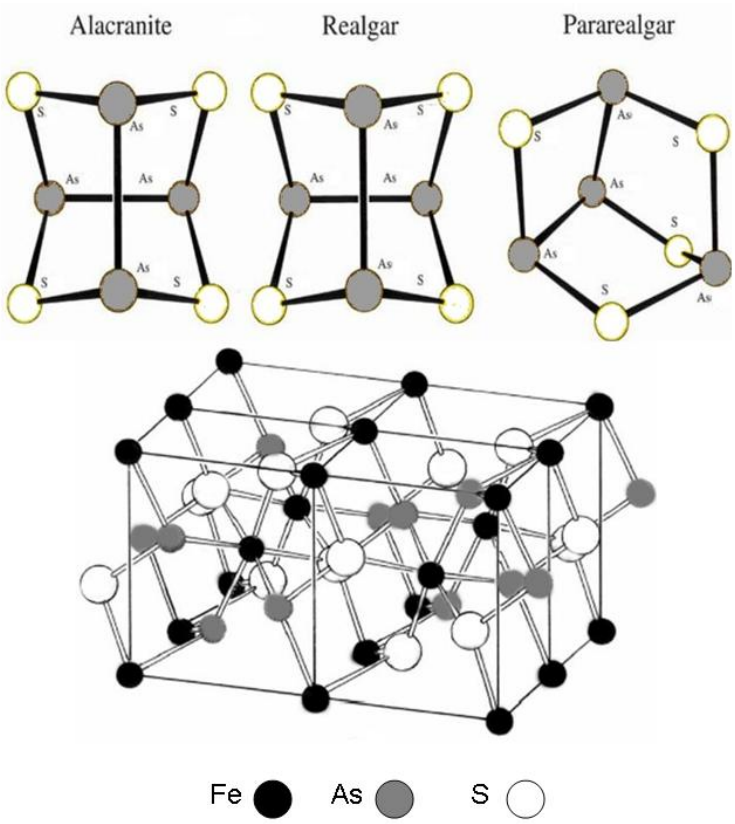


Figure 2.1 Different arsenic mineral crystal structures (after Burns and Percival, 2001; King, 2002).

Table 2.1 Arsenic abundances in different geological sources (Plant et al., 2004; Smedley and Kinniburgh, 2002; Webb, 1978).

Source	As (mg kg^{-1})
Crustal Abundance	1.5
Igneous and Metamorphic Rocks	1-10
Sedimentary Rocks	20-200
Glacial Till/ Alluvial sands	<1-15
Soils†	11

†The data for soils were obtained from a survey of 2600 soils in the Welsh borderlands (Plant et al., 2004).

Fe oxides with associated arsenic on the mineral surface (Acharyya et al., 1999; Anawar et al., 2003). The breakdown of the Fe oxides releases the arsenic into the aquifer, thus making it available to the population in the drinking water (Acharyya et al., 1999; Ahmed et al., 2004; Horneman et al., 2004). Many arsenic contaminated aquifers have elevated arsenic levels resulting from the oxidation of sulphide minerals by the introduction of oxygen into the aquifer by either; 1) the over pumping of the aquifer, 2) irrigation, or 3) high levels of rainfall that create oxidized zones (Rodriguez et al., 2004). Increasing demands for fresh drinking water create more pressure on the world's aquifers, resulting in water removal faster than aquifer recharge rates. This over-pumping of the aquifer introduces oxygen into the aquifer sediments, releasing the arsenic similar to acid rock drainage (ARD). Acid rock drainage is the oxidation of sulphides which releases the bound arsenic and lowers the pH of the environment, allowing arsenic and other metals to remain in solution (Rodriguez et al., 2004). The local areas of oxidation are found in the recharge zones of the aquifer during a dry period in the regional climate. A flushing event, such as a heavy rainfall, then mobilizes the arsenic, increasing regional groundwater arsenic concentrations (Rodriguez et al., 2004).

The Ardkenneth aquifer is an aquitard/aquifer system that has elevated levels of arsenic in the overlying aquitard sediments. The Arkenneth aquifer/aquitard sediments (Snakebite Member) have arsenic levels ranging from 0.3 to 95 $\mu g\ L^{-1}$ (Yan et al., 2000). The arsenic levels in the aquifer are 0.2 to 0.7 $\mu g\ L^{-1}$, within the World Health Organization (WHO) arsenic limits (<10 $\mu g\ L^{-1}$). The risk of arsenic contamination increases with the increase in pumping of the diffusion controlled aquifer (Anawar et al., 2003; Stollenwerk, 2003; Yan et al., 2000). As the water level decreases in the aquifer

the rate of diffusion into the aquifer from the aquitard increases possibly bringing the dissolved arsenic with it. The elevated arsenic levels in the overlying aquitard sequence are located in the redox transition zone (0 to 15 m), suggesting the oxidation of arsenic bearing sulphides is the source (Yan et al., 2000).

2.1.2 Arsenic groundwater geochemistry: Transport and adsorption

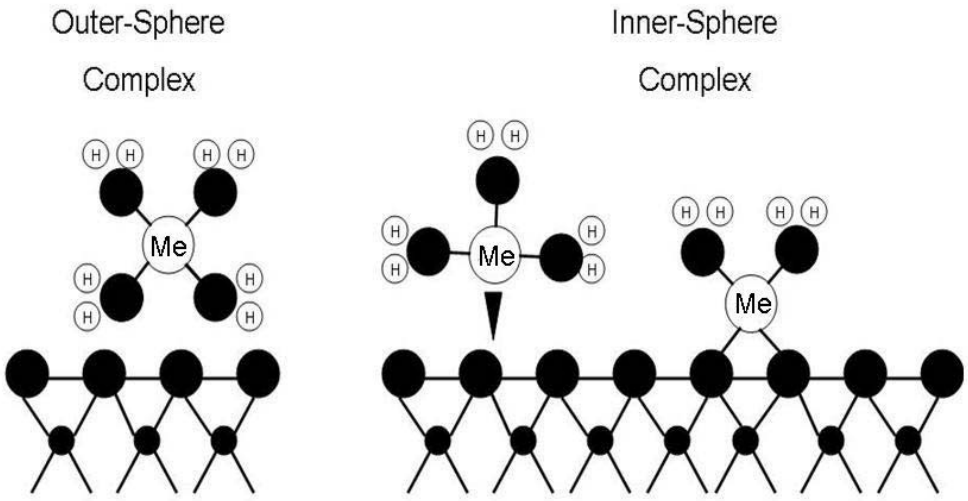
The geochemistry of arsenic is complicated by the five different valencies of the element; -III, -I, 0, III, and V (Goldschmidt, 1954; Plant et al., 2004). Elemental arsenic and arsine [As(-III)] very rarely occur, and are not included in arsenic groundwater geochemistry. Arsenopyrite [As(-I)] is an abundant sulphide, however, it is a mineral and not included in groundwater arsenic geochemistry (Plant et al., 2004). Arsenate [As(V)] and arsenite [As(III)] predominate arsenic groundwater geochemistry, and will be discussed in detail.

The two different species of arsenic in groundwater chemistry are strongly controlled by the Eh and pH conditions of the environment. In oxidizing environments i.e., Eh 0.4 to 1.3 V, As(V) is stable and hydrolyzes to four possible species in the groundwater with increasing pH range; H_3AsO_4, $H_2AsO_3^-$, $HAsO_2^{2-}$, and AsO^{3-}. Fully dissociated arsenic ion is very rare because most groundwater bodies do not have pH values over 11.5 (Foster, 2003; Freeze and Cherry, 1979; Nordstrom and Archer, 2003; Stollenwerk, 2003). Although As(III) is thermodynamically unstable and should oxidize to As(V) in oxidizing conditions, this reaction proceeds very slowly with the reaction half-life ranging from 1 to 3 years in water equilibrated with atmospheric oxygen (Stollenwerk, 2003). However, a study by Yan et al. (2000) indicates the oxidation of As(III) to As(V) is rapid when groundwater is brought to the surface. In reducing

environments i.e., Eh of 0 to -0.8 V, As(III) is stable with the dominant species being fully protonated (H_3AsO_3) over a wide range of pH values (Freeze and Cherry, 1979; Nordstrom and Archer, 2003; Stollenwerk, 2003). The Eh of the Ardkenneth aquitard/aquifer sediments is within the range of -164 mV in the top 3 m to 290 mV at the end of the aquitard sediments at 91.4 m. The Ardkenneth groundwater pH range is 8.1 to 8.4 with a relatively anaerobic Eh (Yan et al., 2000).

Adsorption reactions between arsenic and mineral surfaces dominate dissolved arsenic concentration of arsenic in groundwaters where water supply and quality is an issue (Stollenwerk, 2003). Metal oxides of Fe, Al, and manganese (Mn) are an important source/sink for arsenic in aquifer sediments because of their abundance, chemistry, and ability to coat other particles (Stollenwerk, 2003). There are two accepted mechanisms for the adsorption of arsenic to oxide surfaces: outer-sphere surface complexation and inner-sphere surface complexation (Fig. 2.2). Outer-sphere surface complexation is a non-specific mode of adsorption involving electrostatic attraction between a charged ion and an oppositely charged surface. Common outer-sphere complexes are those formed with anions such as sulphate or selenate. These outer-sphere complexes are very sensitive to ionic strength (I). In contrast, inner-sphere surface complexation is a method of specific adsorption involving the formation of a complex with the mineral surface (Sparks, 2003). Inner-sphere bonds are much stronger then outer-sphere complexation, resulting in stronger adsorption of ions to the mineral surface (Stollenwerk, 2003). Arsenic adsorbs via inner-sphere complexes to mineral surfaces by ligand exchange of OH and OH_2^+ functional groups (Hingston et al., 1972; Stollenwerk, 2003).

A)



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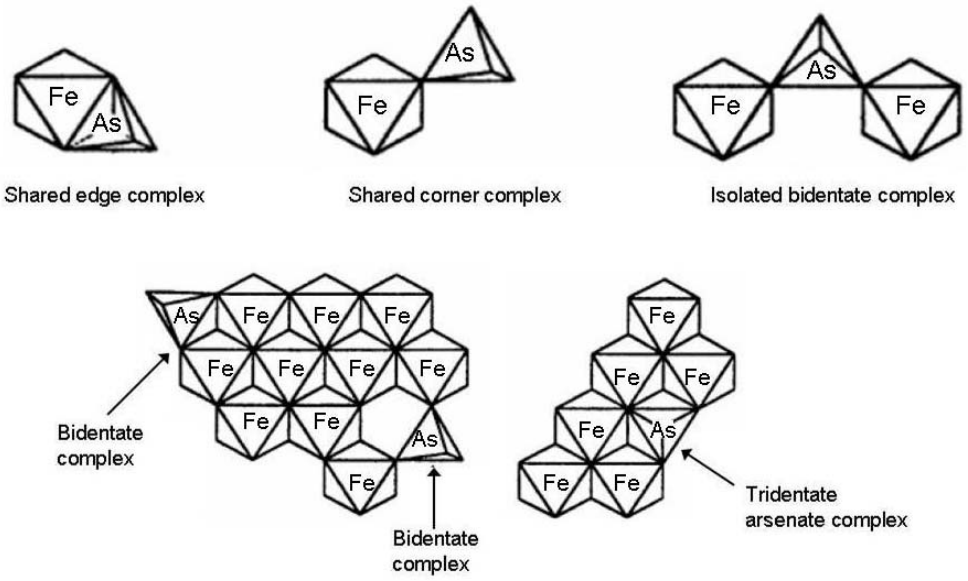

Figure 2.2 A) Schematic drawing of possible metallic adsorption mechanisms to mineral surfaces. B) Inner-sphere arsenic complexes to synthetic Fe oxide minerals (after (Foster, 2003; Manceau, 1995).

Arsenic (V) has a strong affinity for the surfaces of Fe oxides, such as goethite or hematite, at low pH values. Arsenic is desorbed at higher pH values resulting from the electrostatic repulsion because of the increasingly negatively charged Fe oxide surface. The increased adsorption of As(V) at lower pH values is because of more favorable adsorption energies between the more positively charged surface and the negatively charged $H_2AsO_4^-$ molecule, which is the dominant species between pH of 2.2 and 6.9 (Ferguson and Gavis, 1972; Stollenwerk, 2003). When the pH increases above 6.9, $HAsO_4^{-2}$ becomes the dominant species and surface charge becomes less positive, decreasing adsorption capacity. However, at very high pH environments (>10), there are some surface functional groups that will exchange for As(V) (Ferguson and Gavis, 1972; Stollenwerk, 2003). The resulting adsorption isotherm is non-linear and can be approximated by the Freundlich isotherm (Plant et al., 2004). The geometry of adsorbed As(V) to Fe oxides is binuclear with some monodentate inner-sphere complexes (Manning et al., 1998; Stollenwerk, 2003).

As(III) often exists as a neutral $As(OH)_3$ species throughout a wide pH range (4 to 9), making electrostatic outer-sphere interactions not as relevant (Plant et al., 2004). As(III) is adsorbed by Fe oxides over a wide range of pH environments, and since it is neutral in charge the adsorption trend follows a Langmuir isotherm (Plant et al., 2004). As the pH of the system increases above 6.5, As(III) adsorption becomes greater than that of As(V). Sorption of As(III) to Fe oxides becomes greater than As(V) because, at high pH values, the neutral As(III) molecule can readily donate a proton to the surface OH groups (Stollenwerk, 2003). As(III) also forms inner-sphere surface complexes on

goethite, which has been confirmed with EXAFS spectroscopy (Manning et al., 1998; Stollenwerk, 2003).

Aluminum (III) has the same charge and is very close in size to Fe(III). Thus, because of it's similarities to Fe(III), the Al oxide minerals are structurally close to Fe oxides and have arsenic sorption capacities similar to that to Fe oxides (Anderson et al., 1976; Parks, 1965; Stollenwerk, 2003). Although Fe oxides such as goethite adsorb about four times more As(V) than gibbsite, aluminum oxides are still important minerals in arsenic transport and sorption in an aquifer. Arsenic sorption to Al oxide surfaces is not as important for As(III), as As(III) sorption maximizes at a pH of 8, and then decreases at higher pH values (> 8) (Ghosh and Teoh, 1985; Gupta and Chen, 1978).

Manganese oxides also play a role in the sorption of arsenic, and are electron acceptors in the oxidation of As(III) to As(V) (Stollenwerk, 2003). The birnessite group of minerals, common Mn oxides, have negligible sorption of As(V). The negatively charged $H_2AsO_4^-$ is not able to displace the functional group on the negatively charged birnessite surface (Oscarson et al., 1981; Oscarson et al., 1983; Scott and Morgan, 1995). Manganese oxides play a larger role in the sorption of As(III) because of it's neutral charge. As(III) is adsorbed by birnessite at a pH of 7 as the neutral charge on the $H_3AsO_3^0$ molecule results in a lower energy requirement to exchange with the Mn oxide surface functional groups (Oscarson et al., 1983).

Clay minerals such as montmorillonite, kaolinite, illite, halloysite, and chlorite are also able to adsorb arsenic in an aquifer. Clay minerals are composed of layers of alternating Si oxide and Al oxide. Thus, the same properties that control adsorption of arsenic by Al oxide minerals therefore apply to clay minerals (Stollenwerk, 2003). As(V) is adsorbed more readily than As(III) to all clay minerals at pH values < 7. At pH

values > 7 the adsorption levels of both As(V) and As(III) become more comparable (Frost and Griffin, 1977; Lin and Puls, 2000). Halloysite and chlorite adsorb As(V) more readily than kaolinite, illite, and montmorillonite (Lin and Puls, 2000). Adsorption of As(III) by clay minerals has the same controlling factors as Al oxides, so they are not as important as other mineral surfaces for As(III) sorption. However, at high pH values (>7.5), As(III) adsorption can exceed that of As(V) (Frost and Griffin, 1977).

Carbonate minerals could be very important in the control of aqueous arsenic concentrations especially at pH values over 7 (Goldberg and Glaubig, 1988; Oscarson et al., 1981). Arsenic can adsorb to the oxide properties of the carbonate mineral surface. Adsorption of As(V) by carbonates is minimized at a pH of 6 and increases with increasing pH until maximized at a pH of 11 (Stollenwerk, 2003). This pH range for carbonate adsorption of As(V) by carbonates is important for this study as the Ardkenneth groundwater pH ranges from 8.1 to 8.4. Although negligible, As(III) sorption to carbonates occurs at neutral pH values. When allowed to interact with calcite, As(III) was sequestered from the solution and occupied the carbonate sites on the calcite mineral surfaces (Cheng et al., 1999).

The degree of arsenic adsorbed onto a mineral surface greatly depends on the concentration of arsenic in solution and the concentration of available surface sites. However, the presence of competing ions also plays a factor in arsenic adsorption and transport within an aquifer (Stollenwerk, 2003). Common elements found in groundwater can affect arsenic adsorption by competing with adsorption sites on the mineral surface. Competition for adsorption sites is similar to adsorption in that the speciation of the competing anions is controlled by pH, concentration, and binding affinity (Stollenwerk, 2003). Phosphate (P) binds to oxides via inner-sphere surface

complexation similar to that of As(V). The influence of phosphorus [P(V)] on As(III) and As(V) adsorption to Fe oxides is greatly influenced by pH and P(V) concentration (Jain and Loeppert, 2000). As the concentration of P(V) increases adsorption of both As(III) and As(V) to the surface of Fe oxides decreases. The decrease in adsorption of As(V) occurred over the whole pH range because both P(V) and As(V) have the same charge. P(V) competition affects As(III) adsorption slightly at pH values < 9 and adsorption is not affected at all at pH values >9. P(V) competition for adsorption sites does not affect As(III) at high pH values because the neutrally charged $H_3AsO_3^0$ molecule is better able to compete for the adsorption sites with the negatively charged HPO_4^{-2} (Jain and Loeppert, 2000). The addition of P(V) into environmental systems such as soils has been found to increase arsenic mobility (Darland and Inskeep, 1997; Melamed et al., 1995).

Sulphate [S(VI)] can also compete with arsenic adsorption forming outer-sphere complexes with most mineral surfaces. S(VI) has no effect on As(V) adsorption over the whole pH range even though concentrations of sulphate in groundwater are typically much higher than that of arsenic. Sulphate (VI) affects the adsorption of As(III) in acidic environments (pH < 7) (Jain and Loeppert, 2000).

Silica, in the form of silicic acid (H_4SiO_4), can compete for surface complexation sites with arsenic but only affects the adsorption of arsenic to precipitating Fe oxides (Davis et al., 2001; Meng et al., 2000). When the Fe oxides are allowed to age before the addition of silicic acid there is no affect on arsenic adsorption (Swedlund and Webster, 1999). Other elements such as Ca(II) and Mg(II) enhance the adsorption of arsenic to Fe oxides. The cations increase the positive charge on the surface of the metal oxide surface, thus increasing arsenic adsorption (Wilkie and Hering, 1996).

2.2 Environmental Arsenic Cycle

The environmental arsenic cycle includes both the biological and chemical oxidation and reduction of arsenic by diverse organisms (Fig. 2.3). Oxidation of arsenic is important as it can decrease arsenic mobility because As(V) has a stronger affinity for most aquifer sediments than As(III) (Stollenwerk, 2003). Reduction of arsenic is important in the environmental cycle because As(III) is adsorbed to both Mn and Al oxides (Stollenwerk, 2003). Methylation of arsenic does not change the redox state of arsenic, but is an important detoxification step in both higher organisms and microorganisms (Cervantes et al., 1994).

Chemoautotrophs oxidize As(III) by transferring electrons to oxygen, nitrate, and even Fe(III). The initial step of As(III) oxidation begins with the As(III) associating with the oxidase enzyme contacting an embedded Mo(VI) reaction center which begins the electron transport. The electrons in As(III) oxidation follow a chain starting with the arsenite oxidase, continuing to the inner membrane respiratory chain to a cytochrome or an azurin and eventually ending at a terminal oxygen (Silver and Phung, 2005). Almost all bacteria have the genes enabling the oxidation of arsenic in the environment either within the chromosome or on a plasmid (Fig. 2.4) (Cervantes et al., 1994). Chromosomal As(III) oxidation is a detoxification process enabling organisms to tolerate higher concentrations of arsenic in the environment. The plasmid genes coding for the enzyme arsenite oxidase are the *arsAB* genes, also known as *aoxBA*

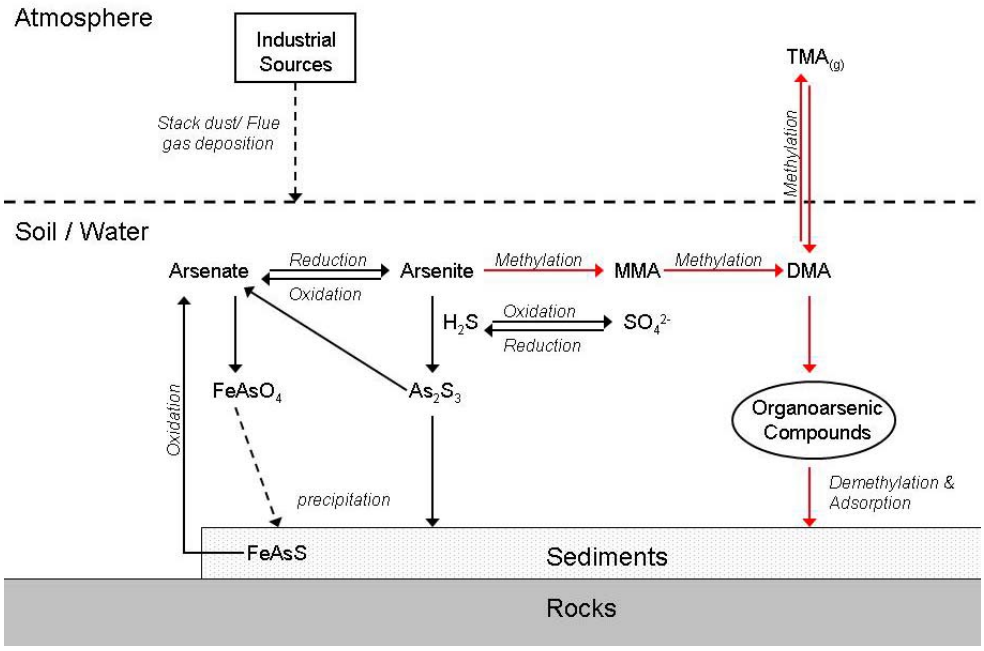

Figure 2.3 Environmental arsenic cycle including both the inorganic and biological (red arrows) speciation of arsenic. The cycle shows the two main sources of arsenic into soil or water: oxidation of sulphides from rocks and sediments and the anthropogenic industrial sources. The biological cycling of arsenic includes the methylation of arsenic to the final trimethylarsenic (TMA) which volatilizes into the atmosphere (after Qin et al., 2006; Roy and Saha, 2002).

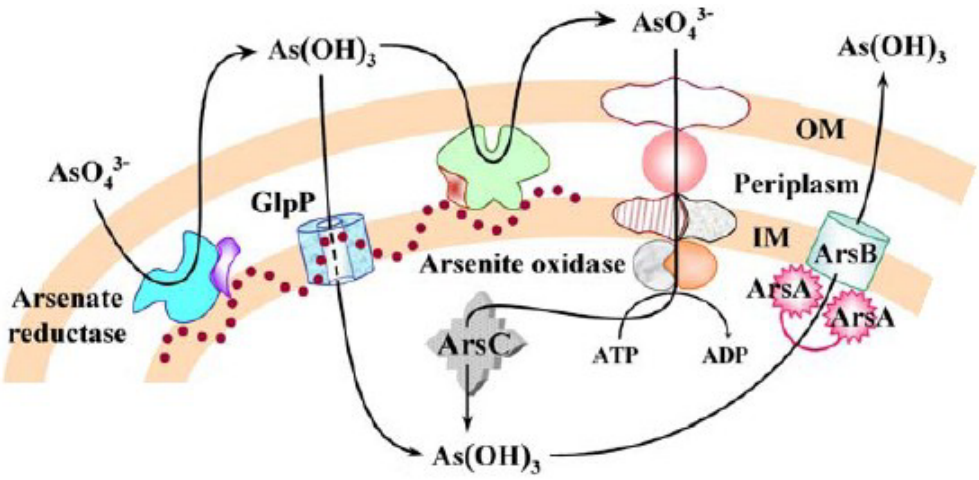

Figure 2.4 Gram negative bacterial cell wall showing both arsenic oxidation and arsenic reduction using an ATPase efflux pump and transporter proteins (modified from Silver and Phung, 2005).

(Silver and Phung, 2005). Chromosomal As(III) oxidation can occur in some *Escherichia coli* strains, some isolated *Alcaligenes* strains such as *Alcaligenes faecalis*, and some cyanobacteria such as *Anabaena variabilis* (Cervantes et al., 1994).

Oxidation of arsenic also can be facilitated by the chemical environment. As(III) is thermodynamically unstable in aerobic conditions, and can rapidly oxidize to As(V) (Yan et al., 2000). Mn oxides are often associated with the oxidation of As(III) and drive the oxidation in a wide range of pH values (Stollenwerk, 2003). The thermodynamic instability of As(III) oxidation to As(V) has a reaction halflife of 1 to 3 years although Yan et al. (2000) observed rapid oxidation when groundwater was brought to the surface. The oxidation of As(III) to As(V) facilitated by birnessite can oxidize up to 80% of As(III) within an hour at a pH ranging between 4 and 8 (Scott and Morgan, 1995). The oxidation of As(III) by Mn oxide surfaces occurs by electron transfer following As(III) adsorption to the mineral surface. The Mn(IV) or Mn(III) is then reduced to Mn(II). The As(V) can be released back into the solution or sometimes becomes incorporated into the Mn oxide structure (Stollenwerk, 2003). The oxidation reaction rate is much quicker in poorly crystalline minerals than more organized minerals (Scott and Morgan, 1995). Oxidation facilitated by Mn oxides can happen independently of the O_2 concentration, making it an important redox control in an aquifer where Mn oxides are often present. Addition of Fe(III) oxides to oxygenated water increases the rate of As(III) oxidation at pH values below 7 (Stollenwerk, 2003). Clays can oxidize As(III) to As(V) in a nitrogen atmosphere (Lin and Puls, 2000). As(III) that was adsorbed to clay mineral surfaces was 100% oxidized after 75 days because of the presence of Fe oxides (Lin and Puls, 2000).

Reduction of As(V) to As(III) by microorganisms is mainly a respiratory function controlled by either chromosomal or plasmid genes (Cervantes et al., 1994). Microorganisms will reduce As(V) during the oxidation of organic matter to hydrogen gas as well as during microbial detoxification of arsenic (Newman et al., 1998). The reduction steps of As(V) start with transfer of electrons from the respiratory electron chain to the arsenate reductase enzyme and then to the arsenate resulting in the release of the As(III) product (Fig. 2.4) (Silver and Phung, 2005). The microbial Fe oxide reduction also can release As(V) into groundwater, such as in the case of the Bengal delta (Akai et al., 2004). As(V) has a high affinity for Fe oxides, when these minerals are dissociated by microbial reduction of the ferric iron [Fe(III)]. The adsorbed As(V) is then released into the groundwater (Acharyya et al., 1999; Plant et al., 2004). Some microorganisms, i.e. *Methanobacterium bryantii*, can reduce As(V) to dimethylarsine (DMA) as a detoxification step (Ehrlich 2002). This DMA production from As(V) requires energy in the form of ATP and utilizes H_2 as the electron donor (Ehrlich 2002). Some organisms are capable of reducing As(V) to As(III) for respiration are *Bacillus arsenicoselenatis*, *Chrysiogenes arsenatis*, and *Desulfomicrobium* strain Ben-RB (Ehrlich 2002).

In the absence of microorganisms As(V) can be reduced chemically in the environment by H_2S. Reduction of As(V) by H_2S is a very rapid reaction and the reaction rates increase with decreasing environmental pH (Rochette et al., 2000). The reduction of As(V) by H_2S forms the intermediate reaction products $H_xAs_3S_{3x}^{-3}$ which will dissociate to form $H_3AsO_3^0$ over a period of several days (Rochette et al., 2000).

Biomethylation of arsenic in the environment is carried out by microorganisms, fungi, and higher organisms including humans (Qin et al., 2006). Humans and other

mammals have the ability to methylate As(III) in the liver, which is then followed by excretion of the methylated species through the urine (Qin et al., 2006). Methylation of As(III) in higher organisms is carried out by products of the *arsM* gene homologues found in some microbial genomes (Qin et al., 2006). The *arsM* gene homologues are widespread throughout nature and have an important role in the global arsenic cycle (Qin et al., 2006). The *arsM* gene encodes for the protein product ArsM or As(III) *S*-adenosylmethyltransferase (SAM). SAM methylates As(III) to monomethylated arsenic (MMA), dimethylated arsenic (DMA), and trimethylated arsenic gas (TMA) (Fig. 2.3). The SAM enzyme methylates arsenite in both bacteria and humans. The reduction of arsenate to arsenite differs from bacteria to higher organisms (Aposhian, 1997).

2.3 Calcite Precipitation

2.3.1 Abiotic calcite precipitation

Calcite is one of the most abundant minerals on earth and composes 3.63% of the earth's crust (Geyssant, 2001). Calcite is a mineral in the carbonate group, closely associated with dolomite, magnesite, rhodochrosite, and siderite. Calcite precipitation occurs chemically in freshwater systems as there is negligible Mg(II) to inhibit the precipitation process (Stumm and Morgan 1995). Precipitation of calcite as a secondary mineral is deposited from hydrothermal waters (140 to 180°C) (Geyssant, 2001). The hydrothermal waters are of high temperature and pressure and therefore have the ability to dissolve $CaCO_3$. When these waters reach the earth's surface, the pressure adjusts to the atmosphere and CO_2 escapes, resulting in calcite veins (Geyssant, 2001).

Calcium carbonate precipitation is controlled by precipitation and dissolution equilibrium reactions. The precipitation dissolution reactions are in turn controlled by the equilibrium constants (Table 2.2) (Stocks-Fischer et al., 1999; Stumm and Morgan, 1995).

$$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3 \downarrow \qquad Ksp = 3.35x10^{-9} \ (25°C)$$

Eq. 2.1

$$CaCO_3 = Ca^{2+} + CO_3^{2-} \qquad \log K_{s0} = -8.480 \ (25°C \text{ and } 1 \text{ bar})$$

Eq. 2.2

Oversaturation of $CaCO_3$ occurs when the aquatic system has an abundant supply of inorganic carbon (usually when the system is open to the atmosphere) so that CO_2 can be dissolved into the aqueous system. Photosynthesis in the upper layers of water bodies removes CO_2 from the water and raises the saturation ratio of $CaCO_3$ (Stumm and Morgan, 1995).

Table 2.2 Equilibrium constants for the dissolution of calcite (Stumm and Morgan 1995).

Reaction		-log K (I=0)		
		5° C	**10° C**	**15° C**
$CaCO_{3\,(s)}$	$(Ca^{2+} + CO_3^{2-})$	8.35	8.36	8.37
$CaCO_{3\,(s)} + H^+$	$(HCO_3^- + Ca^{2+})$	-2.2	-2.13	-2.06

Calcite precipitation depends on both total carbon and pH as seen in Table 2.2. When a system is not in equilibrium with the atmosphere, calcite precipitation can occur at calcium levels lower than those experienced for open systems. In deep aquifer systems the groundwater is not in equilibrium with the atmospheric $CO_{2(g)}$ and thus, the total carbon (C_T) is constant (Stumm and Morgan, 1995):

$$C_T = [CO_{2(aq)}] + [H_2CO_3] + [HCO_3^-] + [CO_3^{2-}]$$

Eq. 2.3

In a closed system where atmospheric $CO_{2(g)}$ is not in equilibrium with the water the P_{CO_2} does not get a chance to rise. As a result carbonate concentrations remain elevated and calcite saturation is reached at lower dissolved concentrations of calcium (White 2003). $CaCO_3$ solubility can be calculated as (Stumm and Morgan, 1995):

$$[Ca^{2+}] = \frac{K_{s0}}{[CO_3^{2-}]} = \frac{K_{s0}}{C_{T\alpha 2}}$$

Eq. 2.4

The α_2 constant is known for any given pH and K_{s0}, the solubility equilibria of $CaCO_3$. Chemical calcite precipitation in fresh water occurs when the system is oversaturated with the dissolved mineral components. The saturation of minerals in an aqueous system is expressed by the saturation index (SI) which is a logarithmic value. The SI value indicates whether a mineral is under or oversaturated in an aqueous system. The SI of calcite can be determined by solubility equilibrium and ion activity product (IAP) (Stumm and Morgan, 1995).

$IAP > K_{s0}$ (oversaturated)

$IAP = K_{s0}$ (saturated)

$$IAP < K_{s0} \text{ (undersaturated)}$$

Undersaturation of a mineral indicates that the mineral will remain in solution unless solution conditions change. Oversaturation of any mineral is indicated by a SI value > 0. Although oversaturation of a mineral does not indicate that it will precipitate, it is more probable it will. The SI for calcite in the Ardkenneth aquifer was determined using the PHREEQc groundwater analysis program, and was determined to be 0.4202. Using the REACTION data block of PHREEQc, the addition of 0.03 M of urea in a 10 step process increases the SI of calcite from 0.4202 to 1.5402. This SI increase increases the probability of chemical calcite precipitation in the groundwater.

DOC, Mg and P can inhibit abiotic $CaCO_3$ precipitation by adsorbing to the surface of the crystal preventing further growth (Langmuir, 1997). Inhibition of $CaCO_3$ crystal growth in the oceans acts to raise the alkalinity and ph of the system as well as Ca(II) ion concentration.

2.3.2 Biotic calcite precipitation

Calcite is one of the most widely known biominerals as it forms the exoskeletons of many of the ocean's invertebrates (Skinner and Jahren, 2004). Examples of such biomineralizing organisms are cyanobacteria, corals, coccoliths, foraminifera, mollusks, and some strains of bacteria (Skinner and Jahren, 2004). This biological form of calcite is so abundant that some strata are composed solely of carbonate shells and exoskeletons (Skinner and Jahren, 2004). The biomineralization of calcite requires certain

environmental conditions, such as adequate amounts of (DIC) and pH to control $CaCO_3$ saturation.

In open systems there is ample amounts of DIC as the water equilibrates with atmospheric $CO_{2(g)}$, which can then dissolve into the water (Hammes, 2003).

$$CO_{2(g)} \leftrightarrow CO_{2(aq)} + H_2O \leftrightarrow H_2CO_{3(aq)} \leftrightarrow HCO_3^{-}{}_{(aq)} + H^+ \leftrightarrow CO_3^{2-}{}_{(aq)} + H^+$$

Eq. 2.5

In closed water systems the system is not in equilibrium with atmospheric CO_2 which could limit DIC (Hammes, 2003). Bacteria can continuously affect the saturation of any given system by changing the concentration of DIC. DIC forms from bacterial metabolism of organic matter, complete oxidation of organic carbon and the electron donor which will result in the formation of CO_2 or HCO_3^- (Hammes, 2003). This increase in CO_2 or HCO_3^- will increase the total carbon (C_T) of the closed system, an important control of $CaCO_3$ precipitation.

Oversaturation of $CaCO_3$ is only the first step in calcite precipitation. Nucleation sites are necessary to begin crystal growth (Hammes, 2003). Bacteria can influence $CaCO_3$ precipitation by changing the regional geochemical conditions and are also able to serve as a nucleation site for crystal formation (Fujita et al., 2000). The microenvironment created by the thin water layer around bacterial cells and the cell surface features of the bacterial membrane contribute to crystal nucleation (Hammes, 2003). The cell surface of both gram positive and gram negative bacteria have a net negative charge resulting from carboxyl and phosphoryl functional groups (Schultze-

Lam et al., 1996). The reaction of binding divalent cations for example Ca(II) and Mg(II), to the negatively charged bacterial cell wall is the beginning of crystal formation (Southam, 2000). $CaCO_3^-$ moieties, or functional groups, start to form on the bacterial cell surface. These moieties are similar to those $CaCO_3$ moieties of the mineral surface (Nilsson and Sternbeck, 1999). The formation of $CaCO_3^-$ functional groups on the bacterial cell surface allow for crystal growth around the bacteria (Schultze-Lam et al., 1996).

Many bacteria can metabolize urea which will produce ammonia and in turn, will increase the pH of the water. The enzyme responsible for urea metabolism or hydrolysis is widespread among microorganisms, so calcite precipitation using urea can take advantage of the natural bacterial populations in any area (Fujita et al., 2000). Bacterial hydrolysis of urea produces ammonium ions in the groundwater (Fujita et al., 2004).

$$H_2NCONH_2 + 2H_2O + H^+ \rightarrow 2NH_4^+ + HCO_3^-$$

Eq. 2.6

The resulting ammonium increases the pH in the groundwater to produce bicarbonate, another source of DIC. Both the pH increase and DIC source are important in $CaCO_3$ precipitation in a closed system (Fujita et al., 2004; Stumm and Morgan, 1995).

Other studies have used urea to promote biogenic $CaCO_3$ precipitation as a remediation strategy. Hammes (2003) used bacterial $CaCO_3$ precipitation to remediate Ca(II)-rich industrial wastewater. Calcium rich wastewater is often a result of bone processing, citric acid production, paper recycling, and landfill leachates (Hammes 2003). By introducing urea into a reactor system containing a sludge with bacteria present, over 80% of the Ca(II) in the wastewater solution was removed by $CaCO_3$

precipitation (Hammes, 2003). Fujita et al (2004) studied ureolytic $CaCO_3$ precipitation as a remediation strategy for strontium (^{90}Sr) contaminated groundwater. Fujita et al (2004) used *Bacillus pasturii* for the precipitation of $CaCO_3$ in a simulated groundwater chemistry based on the Snake River Aquifer in Idaho. The $CaCO_3$ produced in this study incorporated the ^{90}Sr into the calcite crystal matrix because of the high precipitation rates produced by bacterial urea hydrolysis (Fujita et al., 2004).

2.4 Geology of the Ardkenneth Member

The Ardkenneth member is an important continuous aquifer in the South Saskatchewan area (van Everdingen, 1972). The Ardkenneth aquifer is regionally extensive in the South Saskatchewan River valley and extends west to east for approximately 50 kilometers (Caldwell, 1968; van Everdingen, 1968). The Ardkenneth aquifer is one member in an aquifer/aquitard system in the Bearpaw Formation (van Everdingen, 1968).

The Bearpaw Formation is an extensive geologic formation of Upper Cretaceous marine sands and silty clays (North and Caldwell, 1970). In the South Saskatchewan valley the Bearpaw Formation overlies the Oldman Formation and underlies the Eastend Formation (North and Caldwell, 1970). The Bearpaw Formation is divided into 11 members: Aquadell, Cruikshank, Snakebite, Ardkenneth, Beechy, Demaine, Sherrard, Matador, Broderick, Outlook, and an unnamed member (Caldwell, 1968; North and Caldwell, 1970). The Broderick, Sherrard, Beechy, Snakebite and Aquadell members are all composed of thick silty marine clays (Caldwell, 1968). The Outlook, Matador,

Demaine, Ardkenneth, and Cruikshank members are all composed of sands, with local interbedded clay lenses, forming the aquifers of the system (Caldwell, 1968).

The Ardkenneth Member of the Bearpaw Formation is composed of gray-brown fine to very fine poorly sorted, non calcareous, well compacted sands with rusty ironstone concretions (North and Caldwell, 1970). The Ardkenneth aquifer in the Riverhurst area is 20 to 30 m in thickness and underlies the Snakebite Member. The contact between the Ardkenneth and Snakebite members is sharp, unlike the gradual contact with the underlying Beechy Member (van Everdingen, 1972). The Snakebite member is composed of thick marine clays which compile the aquitard overlying the Ardkenneth aquifer (North and Caldwell, 1970; van Everdingen, 1968). The low permeability of the Snakebite member inhibits the movement of water and salts downward into the Ardkenneth aquifer (van Everdingen, 1972). The Snakebite aquitard was found to have arsenic concentrations between 0.3 and 95 $\mu g\ L^{-1}$ with 46.8% As(III) and 53.2% being As(V) (Yan et al., 2000). Although movement of ions between the aquitard and aquifer are diffusion controlled, the Ardkenneth aquifer has much lower arsenic concentrations (0.2 to 0.7 $\mu g\ L^{-1}$).

3.0 GROUNDWATER BIOREACTOR EXPERIMENTS

3.1 Introduction

The majority of arsenic in groundwater is dissolved. Dissolved arsenic is typically found in two oxidation states (As(III) or As(V)) depending on the environmental conditions. This dissolved arsenic is considered mobile, but it may adsorb to oxide minerals such as Fe, Al or Mn-oxides. In addition, $CaCO_3$ can remediate various divalent cations such as Pb(II) and ^{90}Sr from solution, and could have potential for arsenic remediation (Fujita et al., 2004; Rouff et al., 2002). Sorption of Pb(II) onto existing calcite crystals was studied by Rouff et al. (2002) and it was demonstrated that Pb(II) strongly adsorbs to the calcite mineral surface. Ionic strength has no effect on the sorption of Pb(II) which indicates inner-sphere complexation (Rouff et al., 2002). Desorption of Pb(II) is initially much slower than adsorption, so Pb(II) may be effectively immobilized by calcite in environmental systems where this mineral is present (Rouff et al., 2002). Fujita et al. (2004) conducted ureolytic $CaCO_3$ precipitation experiments resulting in the incorporation of ^{90}Sr into the calcite crystal matrix. The enhanced incorporation of ^{90}Sr into the calcite crystal results from the high precipitation rates that can be created by ureolysis by the *B. pasteurii* bacterium (Fujita et al., 2004). The incorporation of ^{90}Sr into the calcite crystal is by substitution; the ^{90}Sr substitutes for the Ca(II) in the crystal lattice forming $SrCO_3$ (Fujita et al., 2004).

Bacteria are well known for their ability to precipitate $CaCO_3$. Stocks-Fischer and Galinat (1999) used *Bacillus pasturii* and urea in different concentrations of $CaCl_2$ to precipitate $CaCO_3$. The $CaCO_3$ precipitation was correlated to the *B. pasturii* growth and indirectly related to ammonia production (Stocks-Fischer et al., 1999). Fujita et al. (2000) conducted similar experiments using bacterial populations cultured from groundwater and a positive control of *Proteus vulgaris*. The medium for $CaCO_3$ precipitation was a nutrient broth (Fujita et al., 2000). Ureolytic $CaCO_3$ precipitation occurs by the hydrolysis of urea which produces ammonia, causing an increase in the pH. The shift in pH causes Ca(II) and CO_3^{2-} to precipitate (Stocks-Fischer et al., 1999). Bacteria can also increase the concentration of DIC and C_T in a system through their metabolic processes resulting in the formation of CO_2 or HCO_3^- (Fujita et al., 2000; Hammes, 2003; Stocks-Fischer et al., 1999). In addition bacteria can act as nucleation sites for crystal growth Fujita et al (2004) found pits similar in shape and size to the *B. pasteurii* in rhombohedral calcite.

The purpose of this study was to precipitate biogenic $CaCO_3$ in groundwater using the natural bacterial population. The groundwater collected from the Ardkenneth aquifer had Ca(II) at 42 mg L^{-1}, which was determined sufficient to precipitate small amounts of $CaCO_3$ using PHREEQC (Parkhurst and Appelo, 1999b). Although the Ardkenneth aquifer is not contaminated with arsenic, the overlying sediments have elevated levels of arsenic in the sediment pore water with concentrations ranging from 0.3 to 95 μg L^{-1}. the elevated arsenic levels are because of the desorption of arsenic on Fe oxides when the alkalinity is raised at depth (Yan et al., 2000). The elevated arsenic levels in the overlaying sediments put the aquifer at risk with increasing demands for fresh water in a diffusion controlled aquitard/aquifer system. A urea concentration of

0.03 *M* was selected by a 10 step REACTION data block using the PHREEQC groundwater modeling software (Parkhurst and Appelo, 1999a; Parkhurst and Appelo, 1999b). The hypothesis of this study was that the addition of 0.03 *M* urea to Ardkenneth groundwater will result in the precipitation of $CaCO_3$. An inoculant was not necessary as the natural bacterial population was hypothesized to be able to adequately hydrolyze the added urea.

3.2 Materials and Methods

3.2.1 Bioreactor experiments

Groundwater. Groundwater from the Ardkenneth aquifer was collected at the Riverhurst piezometers installed by the Saskatchewan Research Council (SRC) (1962-64). A total of 28 piezometers were installed in the Riverhurst and Lucky Lake area of Southern Saskatchewan between 1962 and 1964. The piezometers were installed in a triangular pattern on both the west and east side of the valley. Groundwater collected from piezometer 4A was used for this study. Groundwater chemistry was analyzed after the two collection dates in April of 2005 and May of 2006 by SRC Analytical. Field pH of the groundwater was taken using a glass pH electrode and an Accumet Research AR20 bench top pH/conductivity meter (Fischer Scientific, Canada). The pH electrode was calibrated before each reading with pH 4, 7 and 10 buffers (VWR, Canada). Groundwater samples sent to SRC for elemental analysis were not filtered in the field, and samples for metal analysis were preserved in the field with nitric acid. SRC Analytical stored the samples between 7 to 10 days before analysis. Major Ions and arsenic were analyzed using inductively coupled plasma mass spectrometry (ICP-MS). Metals were analyzed using inductively couples plasma atomic emission spectrometry

(ICP-AES). Both ICP-MS and ICP-AES have detections limits for concentrations in the ng L^{-1} range. DOC was analyzed using the Dorhrmann Phoenix Six 8000 carbon analyzer. Chloride was analyzed an Aquachem colorimetric method, and Alkalinity was determined by auto-titration method using a Man-tech PC titration system.

Cycle duration and sampling. Each cycle for each separate bioreactor experiment was five days in length. This cycle duration was determined by ureolytic organism hydrolysis times for positive growth in urea test broth, consistent with results from Hammes (2003). Urea (0.3 mol L^{-1} or 0.06 mol L^{-1}) was added to the bioreactors and allowed to incubate at 5°C at an Eh normal hydrogen electrode (NHE) potential of 200 to 300 mV for five days. The NHE also known as the Standard Hydrogen Electrode is the thermodynamic reference point for all potential measurements where the potential difference is zero based on the Nernst equation. The hydrogen electrode is based on the redox half cell (Rayner-Canham, 2000b):

$$2H^{+}_{(aq)} + 2e^{-} \rightarrow H_{2(g)}$$

Eq. 3.1

Potentials of any other electrodes are then compared to the normal hydrogen electrode. Samples for analysis were taken after the incubation period. The Eh electrodes were calibrated before each reading with a 80 to 90 mV and a 260 to 270 mV buffer made by adding 0.05 g of quinhydrone ($C_{12}H_1O_4$) (Alfa Aesar ®, USA) to pH 7 and 4 buffers respectively.

Urea concentrations and Ca(II) analysis. Chemical grade urea from J.T. Baker was used for the bioreactor experiments. A 5 *M* urea stock solutions was made, filter sterilized using a 0.2 μm filter and stored in a sterile Nalgene container. Urea

concentrations were selected based on previous remediation studies by Hammes et al. (2003). The urea concentrations selected for this study were also confirmed to precipitate $CaCO_3$ by Fujita et al. (2003), Fujita et al. (2000), and Stocks-Fischer et al. (1999) and geochemical modeling. Urea concentration modeling was performed using the PHREEQC groundwater modeling program, with the REACTION data block adding urea in a 10 step process (Parkhurst and Appelo, 1999b). Using the saturation index (SI) for calcium as the defining parameter, 0.03 *M* was selected as the concentration where the SI for $CaCO_3$ reached its peak.

Calcium was monitored with ThermoOrion Ca(II) IonPlus ion selective electrode (ISE). The ISE membrane determines the activity of hydrated, uncomplexed Ca(II) ions in the solution by determining the potential difference across the membrane. The mV output reading was then converted to mg L^{-1} using the $\log_{10}$ mol Ca(II) and the mV reading. The Ca(II) concentration based on activities determined by the Ca(II) electrode was verified using atomic adsorption (AA) on 12 randomly selected samples (Appendix A).

Arsenic analysis. Arsenic was analyzed using the Millennium Excalibur hydride generation atomic florescence spectroscopy (AFS). The Millenium AFS utilizes a continuous flow hydride generation system for the detection of arsenic. Three solutions are required for arsenic analysis: 1) sodium tetrahydroborate (1.5% m v^{-1} $NaBH_4$) (J.T. Baker, USA) in 0.1 mol L^{-1} NaOH, 2) 1 L of reagent blank composed of 300 mL Omni*Trace*® HCL (37%), 680 mL Milli-Q® ultrapure water, and 20mL of a 50% m v^{-1} potassium iodide (KI) solution (BDH) and 10% m v^{-1} ascorbic acid ($C_6H_8O_6$) (Alfa Aesar®, USA), and 3) prepared samples. Samples were prepared two ways: filtered through a 0.45 μm syringe filter (VWR nylon syringe filter) for dissolved arsenic and

unfiltered for total arsenic. To each 9 mL total and dissolved arsenic sample 6 mL of pure Omni*Trace*® HCl (37%), 4.5 mL of Milli-Q® ultrapure water, and 0.4 mL 3.01 *M* potassium iodide (KI) and 0.57 *M* ascorbic acid ($C_6H_8O_6$) solution was added. The potassium iodide solution reduces all arsenic in the solution to As(III) which then forms the hydride complex necessary for spectrophotometric analysis.

Dissolved and total organic carbon analysis. DOC and total organic carbon (TOC) were analyzed to ensure that the Eh was being controlled and no bacterial blooms were present in the bioreactor system. DOC and TOC were analyzed using the Shimadzu TOC analyzer. A standard curve was prepared using known organic carbon (OC) concentrations made from a 1000 mg L^{-1} OC stock solution. The OC stock solution contained 1.0625 g of potassium hydrogen phthalate ($C_8H_5O_4K$) (Fisher Scientific) dissolved in 500 mL Milli-Q® ultrapure water. DOC samples were syringe filtered using a 0.45 μm pore filter, where the TOC samples were not filtered and treated with 50 μL of 6 *M* HCl acid. To correct for any changes in DOC or TOC upon urea addition, a solution of 0.03 *M* urea with Milli-Q® ultrapure water and a 0.03 *M* urea solution in Ardkenneth groundwater were also analyzed for OC each time. The Shimadzu TOC analyzer determined the non-purgeable OC which refers to the non volatile OC content of a sample. A combustion tube filled with an oxidation catalyst and heated to 680°C where the sample undergoes combustion and all OC is decomposed to CO_2. The sample combustion product then runs through a halogen scrubber in a sample cell set within a non-dispersive infrared gas analyzer where the CO_2 is detected. The non-dispersive infrared gas analyzer generates a peak which the TOC analyzer software can then convert to a TOC measurement in mg L^{-1}.

3.2.1.1 Flow-through bioreactors

The first two bioreactor experiments were a flow-through system designed to replenish the system with fresh groundwater after each cycle period. Three 250 mL Wheatman Celstir water jacketed flasks were set up, each containing 600 mL Ardkenneth groundwater with urea at a concentration of 0.03 mol L^{-1}. The three control bioreactors were identical with no urea added. The bioreactors were incubated at a temperature of 4° C (+/- 1°C) for 5 d. Eh and free Ca(II) were monitored continuously during each cycle. After the cycle was completed groundwater was collected from each of the six bioreactors for analysis of DOC, TOC, and arsenic. Electrodes were re-calibrated during each sampling to correct for any drift that may have occurred during the 5 day cycle. After the first bioreactor experiment the bioreactors were sparged with N_2 gas to control the Eh of the system. Both control and urea treated bioreactors were sparged when needed, when the Eh was higher than 300 mV, for 5 minutes at a time.

3.2.1.2 Non flow-through bioreactors

The third and fourth bioreactor experiments were closed system where groundwater was not refreshed during the experiment. In the third bioreactor experiment, each flask contained 600 mL of groundwater with three experimental groups of control, 0.03 mol L^{-1}, and 0.06 mol L^{-1} urea with a 10 d incubation period. The Eh was controlled by sparging the bioreactor system with N_2 gas as needed and was monitored daily with platinum redox electrodes. Analysis of DOC, TOC, and arsenic was done on the initial (Day 0) and final (Day 10) groundwater samples. Free Ca(II) was also measured using the Ca(II) ISE at the initial (Day 0) and final (Day 10) day of the experiment.

The fourth and final bioreactor experiment had two experimental groups, control and 0.03 mol L^{-1} urea, and ran for a duration of 40 d. Analysis of DOC, TOC, and total arsenic were done on both the initial (Day 0) and final (Day 40) samples of the groundwater. The free Ca(II) was measured every 5 days using the Ca(II) ISE. The electrodes were calibrated to account for any drift when free Ca(II) was measured (r^2= 0.997 to 0.998).

3.3 Statistical Analysis

Data was analyzed using a two-way general linear model (GLM) using MINITAB 11. Multiple comparisons were performed using least significant difference (LSD). All analysis used a α=0.05. The response, or single dependant variable for the GLM multiple regression was the concentration of the variable being tested, such as the concentration of Ca(II), arsenic, or OC. Factors in the GLM were the treatment, cycles and sample preparation (total and dissolved). Where necessary non-normal data was log transformed to normalize the data set, though this was often not needed. Data sets that needed log transformation were the Eh data from the second bioreactor experiment and the DOC/TOC and Eh data from the fourth bioreactor experiment.

3.4 Results

3.4.1 Groundwater analysis

The groundwater collected from piezometer 4A was analyzed by SRC analytical for major cations, anions, dissolved organic carbon (DOC) and arsenic (Table 3.1). The average composition of the groundwater was: pH 8.29 (+/- 0.1); dissolved organic carbon (DOC) 4.6 to 6.3 mg L^{-1}; free Ca(II) 36 to 42 mg L^{-1}; and total arsenic 0.2 to 0.7

μg L^{-1}. The weak interactions of As(V) at higher pH environments have major environmental consequences as environments such as the Ardkenneth aquifer have groundwater pH around 8.5, well within the desorption range of 8 to 9.

3.4.2 Bioreactor experiments

3.4.2.1 Flow-through Bioreactors: Experiment #1

The Eh was significantly higher in the 0.03 M urea treated group ($p=0.00$) in the first experiment than the control group (Fig. 3.1). The Eh remained stable in the control group, ranging from 226 mV to 277 mV. In the treated group the Eh was high initially at 466 mV and lowered to a range of 350 to 360 mV over the course of the experiment. The DOC of the treated groundwater in the first experiment was significantly higher at 22 mg L-1 (SD= 1.5), compared to the control group at 10 mg L-1 (SD= 1.1) ($p<0.01$; Fig. 3.1). The DOC of both the control and urea-treated groups remained stable throughout the five experimental cycles. The pH remained stable at a value of 8.5 to 8.6.

Table 3.1 Ardkenneth groundwater chemistry for two collection dates; April 2005and May 2006. Elemental analysis by Saskatchewan Research Council (SRC) Analytical.

Element	**Concentration (**mg L^{-1})	
	April 2005	May 2006
Al	0.005	0.005
As	0.0002	0.0007
Ba	0.25	0.25
C	364	388
B	3.4	3.3
Cd	0.001	0.001
Ca	42	36
Cl	2700	2870
Cu	0.001	0.001
Fe	0.6	1.45
Pb	0.002	0.002
Mg	8.7	9.0
Mn	0.031	0.034
Ni	0.001	0.001
DOC	4.6	6.3
P	0.64	0.38
K	10	7
Si	6.5	4.4
Ag	0.001	0.001
Na	1770	1990
Sr	0.7	0.69
S	0.2	0.2
Zn	0.005	0.28

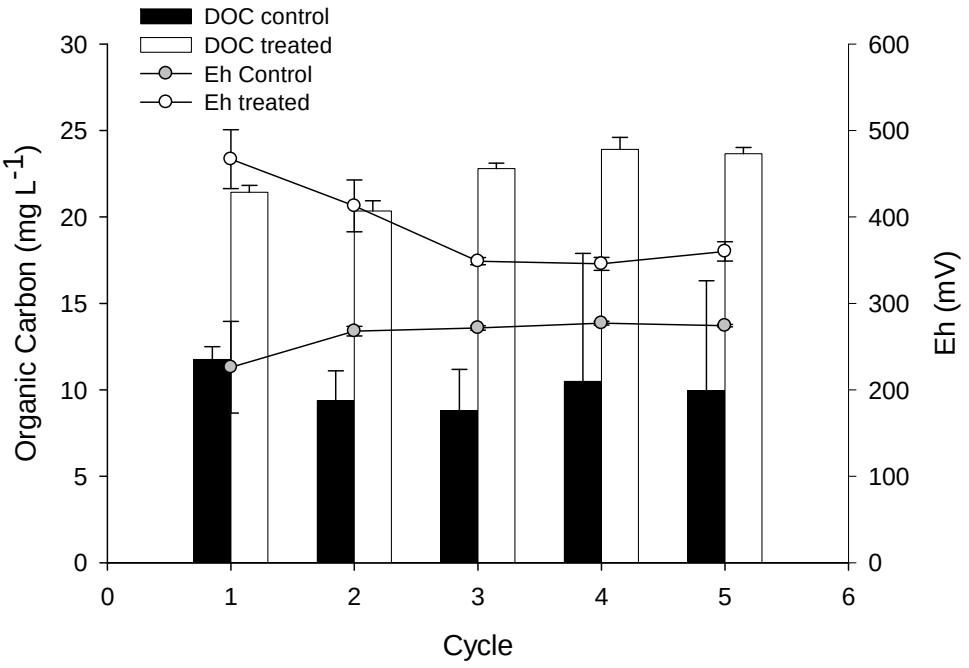

Figure 3.1 The dissolved organic carbon (mg L^{-1}) and Eh as normal hydrogen electrode (mV) data over time for the first bioreactor experiment. The DOC data is an average of each treatment for the whole five cycle experiment. The Eh electrode data is an average of the two different urea concentrations for each cycle of the experiment. Error bars represent the standard deviations.

3.4.2.2 Flow-through Bioreactors: Experiment #2

The second bioreactor experiment sparged the system with N_2 gas to control the Eh in each bioreactor flask. Sparging the bioreactor system with N_2 gas lowered the overall DOC and Eh in the urea treated groups (Table 3.2). There was no difference between the DOC and TOC data for experiment #2 (p=0.1) so TOC is not reported. The DOC in the sparged experiment in the urea treated bioreactors at 16 mg L^{-1} (SD= 0.5) was much lower than the first bioreactor experiment at 22 mg L^{-1} (SD= 1.5) (Fig 3.1 and Table 3.2). The DOC, however, was higher at 16 mg L^{-1} (SD= 0.5) in the treated group than that of the untreated control group at 12 mg L^{-1} (SD=0.3; p=<0.01; Table 3.2).

The Eh was lower in the second experiment with sparging. The Eh was approximately 200 mV (SD=81) in the treated group of the second experiment, much lower when compared to 400 mV (SD= 52) in the treated group of the first bioreactor experiment. Sparging the bioreactor system with N_2 gas effectively kept the Eh within the desired range for this experiment. Similar to the first bioreactor experiment the pH remained stable at a value of 8.6.

The free Ca(II) (μmol) were significantly higher in the control group (p<0.01). The average arsenic was significantly higher in the unfiltered rather than filtered samples in all four of the experimental cycles (p<0.01) so that particulate arsenic was calculated. The average arsenic, over the duration of the experiment, did not however, decrease between the control and treated groups of groundwater samples in both the dissolved (p=0.27) or particulate samples (p=0.058; Fig. 3.2).

Table 3.2 Dissolved organic carbon (DOC) and Eh (mV) results from a one-way general linear model (GLM) comparing the mean control and urea treated samples from the second bioreactor experiment with a 95% confidence interval (n=3).

Analyte	**Mean**	**Standard deviation**	*p* values
DOC control	12.1	1.0	<0.001
DOC + urea	16.5	1.0	
Eh control	207	1.0	<0.001
Eh + urea	223	1.0	

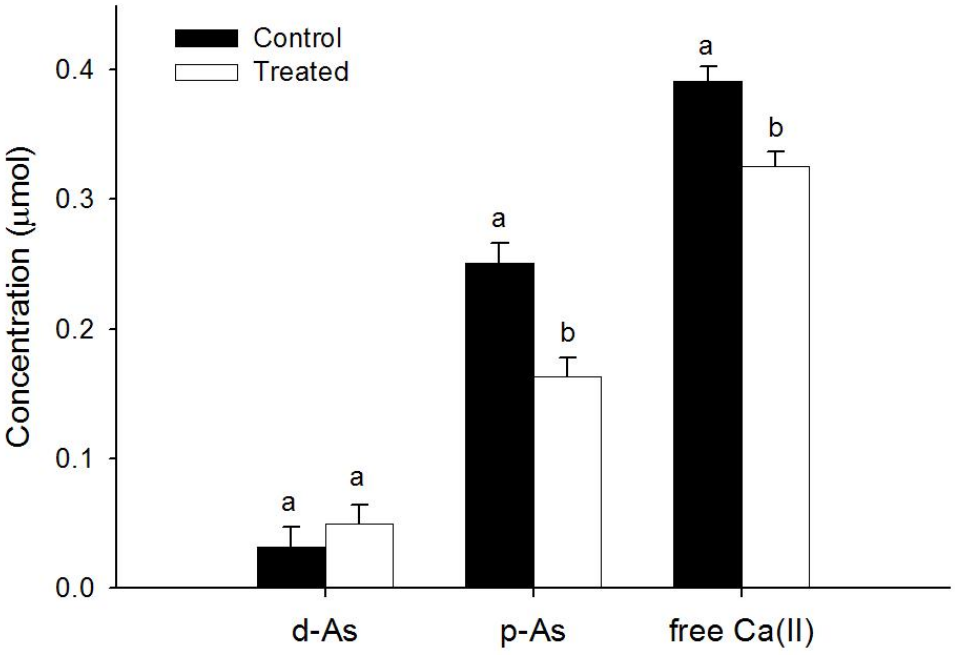

Figure 3.2 The mean dissolved arsenic (d-As), particulate arsenic (p-As), and free Ca(II) for the control and urea treated groups for the second experiment. The second experiment sparged the bioreactors with N_2 gas. Means with the same letter are not significantly different according to a one-way general linear model (GLM). Error bars represent standard deviations (n=3).

3.4.2.3 Non Flow-through Bioreactors: Experiment #3

To increase $CaCO_3$ precipitation the third bioreactor experiment, three urea treated experimental groups were duplicated; 0 mol L^{-1}, 0.03 mol L^{-1}, and 0.06 mol L^{-1}. There was no difference between treatments for the free Ca(II) in the third experiment (p=0.557) or between the initial and final measurements (p=0.860; Fig. 3.3). Similarly, there was no difference in the arsenic concentrations among the different treatments (p=0.948) or between the filtered and unfiltered samples (p=0.596; Fig. 3.3).

The Eh was monitored daily for the two urea treatment groups but not the control group. However, the control group was also sparged with N_2 gas when the treated groups were sparged. There was no difference between the DOC and TOC data for the third experiment (p=0.303) so that TOC data was not reported. The 0.03 M urea L^{-1} treatment group had 6.5 mg L^{-1} OC (SD=0.003; p=0.01). The DOC data was higher in both treatments (p=0.013) compared to the control (Table 3.3). The Eh of the 0.03 mol L^{-1} urea treatment was significantly higher (p<0.01) than that of the 0.06 mol L^{-1} urea treatment for the 10 d experiment. The pH had a an increase from the initial measurement of 8.5 to a value of 8.7 over the 10 d incubation period.

3.4.2.4 Non Flow-through Bioreactors: Experiment #4

The fourth bioreactor experiment had only two treatments of urea (0 mol L^{-1}, and 0.03 mol L^{-1}) and an incubation duration of 40 d which is four times longer than the third experiment. There was no difference between the two treatments in the free Ca(II) (p=0.81). However, the concentration of free Ca(II) was significantly lower at the end of the experiment than initially (p<0.01; Fig 3.4).

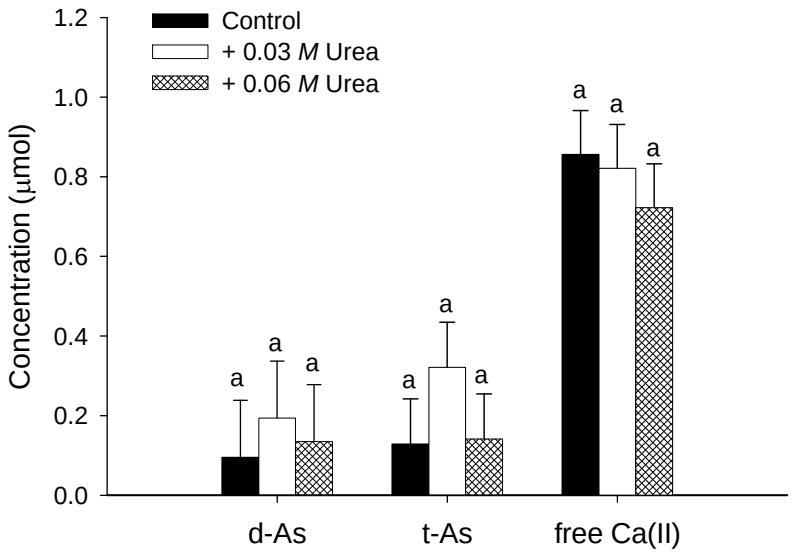

Figure 3.3 The mean dissolved arsenic (d-As), total arsenic (t-As), and free Ca(II) for both the control, 0.03 *M* urea, and 0.06 *M* urea treated groups for the third bioreactor experiment. Means after 10 d with the same letter are not significantly different according to a one-way general linear model (GLM). Error bars represent standard deviations (n=2).

Table 3.3 Dissolved organic carbon (DOC) and Eh (mV) results from a one-way general linear model (GLM) comparing the mean control, 0.03 *M* , and 0.06 *M* urea treated samples from the third bioreactor experiment (n=2).

Analyte	**Mean**	**Standard deviation**	*p* values
DOC control	3	0.9	
DOC + 0.03 *M* urea	6	0.9	0.013
DOC + 0.06 *M* urea	7	0.9	
Eh control	n/a	n/a	
Eh + 0.03 *M* urea	600	1.0	<0.001
Eh + 0.06 *M* urea	208	1.0	

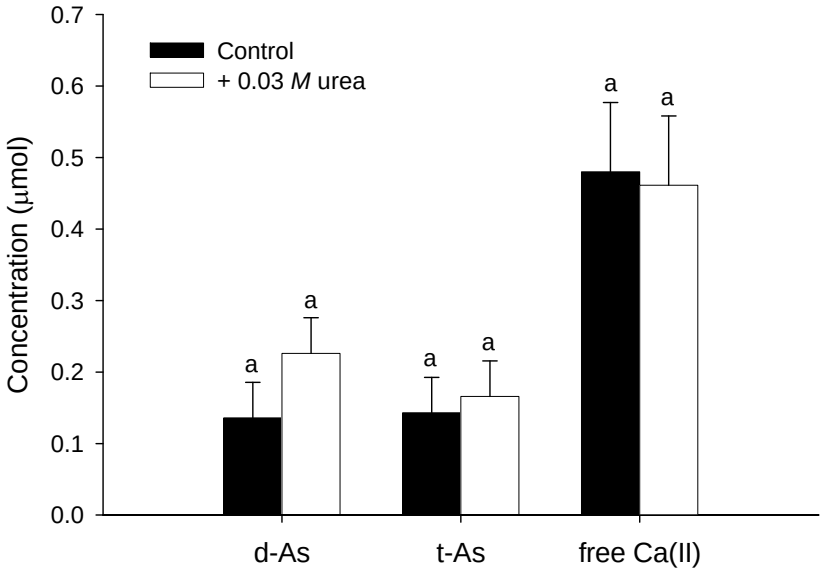

Figure 3.4 The mean dissolved arsenic (d-As), total arsenic (t-As), and free Ca(II) for both the control and 0.03 *M* urea treated groups for the fourth bioreactor experiment. Means with the same letter are not significantly different according to a one-way general linear model (GLM). Error bars represent standard deviations.

There was no difference between the total and dissolved arsenic concentrations, so particulate arsenic was not calculated (p=0.593). There was no difference in the arsenic concentrations between the control and 0.03 *M* urea treated samples (p=0.262; Fig. 3.4).

Similar to the third bioreactor experiment, there was no difference in the DOC and TOC between each treatment, and thus, the TOC data was not reported (p=0.853; Table 3.4). The DOC for the 0.03 mol L^{-1} treatment group was 5.2 mg L^{-1} (SD=3.4), slightly higher than that of the third bioreactor experiment. The DOC was higher in the control group then the 0.03 *M* urea treated group (p=0.013). There was a difference in Eh between the two treatments, the control being 345 mV (SD= 126) and the treated group being 336 mV (SD=103) (p<0.01). Similar to the other bioreactor experiments, with the exception of the third experiment, the pH remained stable over the 40 d incubation period at a value of 8.5.

Table 3.4 Dissolved organic carbon (DOC) and Eh (mV) results from a one-way general linear model (GLM) comparing the mean control and 0.03 *M* urea treated samples from the fourth bioreactor experiment.

Analyte	**Mean**	**Standard deviation**	*p* values
DOC control	9	1.0	0.013
DOC + 0.03 *M* urea	5	1.0	
Eh control	345	1.0	<0.001
Eh + 0.03 *M* urea	336	1.0	

3.5 Discussion

Free Ca(II) decreased only in the second bioreactor experiment. There was no difference between the free Ca(II) in other experiments even with changing the system to a non flow-through system and increasing the incubation duration. Hammes (2003) observed a decrease of over 80% of free Ca(II) by $CaCO_3$ precipitation from urea hydrolysis. In that study, the Ca(II) rich wastewater experiments were conducted using bacteria at room temperature with either a 24 h or a 8 h residence time after the first week. Significant Ca(II) decreases in wastewater with soluble Ca(II) concentrations of 489 mg L^{-1} will be more readily noticed compared to a natural groundwater with 36 to 42 mg L^{-1} Ca(II) (Hammes, 2003). Fujita et al. (2004) conducted a similar ureolytic $CaCO_3$ remediation study precipitating $CaCO_3$ in a synthetic groundwater medium using both a natural groundwater bacterial population and a positive control. Fujita et al. (2004) were successful in $CaCO_3$ precipitation, however, the synthetic groundwater had levels at 199 mg L^{-1} Ca(II) which is much higher than the Ardkenneth groundwater (Fujita et al., 2004).

Dissolved arsenic only decreased in the second bioreactor experiment. The second bioreactor experiment was also the only one with significant decrease in free Ca(II). These results may suggest that precipitation of $CaCO_3$ does sequester dissolved arsenic. Subsequently there was no precipitate for analysis. Remediation studies by Rouff and Reeder (2002) observed significant removal of a Pb(II) from aqueous solution by sorption onto $CaCO_3$. Although the Pb(II) removal was significant, the $CaCO_3$ was not biogenic in origin and thus would have different surface and adsorption properties than the $CaCO_3$ possibly produced in the bioreactor experiments (Rouff et al., 2002).

A flow-through bioreactor system was used initially to optimize the bacterial population that was actively involved in $CaCO_3$ precipitation. If a bacterial cell as the nucleation site of crystal growth the cell would be weighted down and eventually sink to the bottom of the bioreactor flask. The unidirectional flow of the flow-through bioreactor system would then rid the bioreactor system of other bacterial cells not participating in $CaCO_3$ precipitation and increase the bacterial population involved in the nucleation of $CaCO_3$ crystal growth. The flow-though bioreactor system did not optimize $CaCO_3$ precipitating bacterial populations as no visible $CaCO_3$ was formed at all in any bioreactor experiment. Optimization of $CaCO_3$ precipitating cultures would have to be done in another way, such as inoculation of ureolytic active cultures.

Sparging the bioreactor system with N_2 gas controlled the Eh and limited any bacterial bloom. Sparging the bioreactor systems also reduced the OC present in the reactors and typically lowered the Eh values when compared to the initial bioreactor experiment. In the first bioreactor experiment the initial spike in Eh leveled off over time as a result of bacterial consumption of O_2 during bacterial metabolism (Fig 3.1). The spike in Eh in the first bioreactor experiment may have contributed to the visible bio-film growth on the borosilicate glass beads in the bottom of the treated reactors. The Eh of the control group was stable in all bioreactor experiments compared to the treated group, likely because of a lack of bacterial production spike as no urea was present to stimulate bacteria. Other studies with ureolytic $CaCO_3$ formation conducted experiments in the open atmosphere at room temperature. Consequently they did not have Eh regulatory issues when the experiments were carried out open to the atmosphere (Fujita et al., 2000; Stocks-Fischer et al., 1999; Warren et al., 2001).

The OC was higher in the treated groups compared to the control groups in all four bioreactor experiments. The urea [$CO(NH_2)_2$] treatment adds approximately 10 to 40 mg L^{-1} C to the system, affecting the OC measurements. This increase in OC measurements for the treated groups was corrected for by adding urea to MilliQ® ultrapure water and subtracting that concentration from the treated measurements on the Shimadzu TOC analyzer. After correcting for the addition of urea the OC was still higher in the treated groups because of bacterial growth and OC production, as a visible biofilm was present in the bioreactors. There was no significant difference in OC for both sample preparations, filtered (DOC) and unfiltered (TOC), so particulate OC was not determined and TOC data was not reported.

There was variability in all electrode measurements for each bioreactor experiment. The Eh data varied throughout all experiments, with very high standard deviations. This variation of Eh could be a result of the crystallization of the reference solution when it was kept in the 4°C groundwater for the duration of the experiment. The 4 M KCl reference solution contains 298.2 g L^{-1} of KCl. However, the solubility of KCl at 4°C is 289.6 g L^{-1} leaving 8.5 g of KCl out of the solution (Chang, 1998). The first two bioreactor experiments allowed fresh groundwater to flow into the system, which also could contribute to Eh variability. The Eh electrodes were functioning during the influx of fresh Ardkenneth groundwater into the bioreactor system as they were hooked up to a data logger. The addition of fresh water to the system in the first two bioreactor experiments introduced O_2 into the bioreactors and raised the Eh. Previous $CaCO_3$ precipitation studies were conducted in open atmosphere with no addition of fresh groundwater, avoiding any Eh issues (Fujita et al., 2004; Hammes et al., 2003; Stocks-Fischer et al., 1999). To control the Eh, the third and fourth bioreactor

experiments were conducted in a closed bioreactor system. The groundwater was brought to the surface, equilibrating the CO_2 with the atmosphere, however over time the CO_2 would be utilized by the bacteria during the course of the closed bioreactor experiments.

The variation of Ca(II) data could be because the free Ca(II) electrode membrane has a limited life-span, and is very sensitive to temperature. The ISE measurement will change with membrane age. Although PVC membrane's have a longer life than other membrane materials, membrane replacement is required over time (Ahmad-Bitar et al., 1983; Arnold and Meyerhoff, 1984). Similar to the Eh electrode, prolonged submersion of the electrode in 4°C groundwater crystallizes the reference solution, altering the data. The Ca(II) ISE reference solution contains approximately 162.95 g L^{-1} KNO_3. However, the solubility of KNO_3 at 4°C is around 145 g L^{-1} and therefore, approximately 17.95 g of KNO_3 will remain out of solution (Chang, 1998). After the second experiment the Ca(II) ISE was used at five day intervals and was removed from the 4°C groundwater after sampling. Although the electrodes were calibrated on a regular basis, some discrepancy occurred in all the electrode data. Previous studies precipitating $CaCO_3$ did not measure decreasing free Ca(II) but rather direct measurements of the precipitate (Fujita et al., 2000; Stocks-Fischer et al., 1999; Warren et al., 2001). Precipitates were identified by X-ray diffraction (XRD) to confirm the presence of $CaCO_3$ as the precipitation product (Fujita et al., 2000; Stocks-Fischer et al., 1999; Warren et al., 2001). This study produced no precipitates for analysis. Investigations into ureolytic $CaCO_3$ precipitation for remediation of ^{90}Sr by Fujita et al. (2004) measured ^{90}Sr and Ca(II) in both aqueous and solid samples using inductively coupled plasma with atomic

emission spectrophotometric detection. Hammes (2003) measured the Ca(II) removal from wastewater using flame atomic absorption spectrometry (FAAS).

The results of this study suggest that indigenous microorganisms in a bioreactor system do not optimally perform ureolytic $CaCO_3$ precipitation. Further studies with ureolytic $CaCO_3$ precipitation should consider a medium, such as groundwater, with high Ca(II) concentrations.

Biogenic calcite precipitation can be facilitated by the addition of urea to the system. Groundwater treated with 0.03 M urea did not consistently decrease free Ca(II) or arsenic from the groundwater using a bioreactor system. This inconsistency in the decrease of Ca(II) and arsenic may be because of the low Ca(II) concentration of the groundwater in that decreases are not as easily noticeable unlike a solution with a very high Ca(II) concentration.

4.0 INOCULATION EXPERIMENTS

4.1 Introduction

The simple addition of 0.03 μg L^{-1} urea to Ardkenneth groundwater did not result in a precipitate. This absence in $CaCO_3$ precipitation is a result of the low Ca(II) concentration in the groundwater which would make any decreases in Ca(II) insignificant. Also the microorganisms involved may precipitate out $CaCO_3$ at slower rates because of a low Ca(II) concentration.

Different ureolytic isolates have been found to produce very different $CaCO_3$ crystal morphologies at varying rates (Hammes et al., 2003). Some strains that Hammes (2003) identified took as long as 5 to 10 days for $CaCO_3$ crystallization and formed spherical crystal shapes while other strains formed larger light brown crystals in 20 to 48 hours. All of the isolates identified by Hammes (2003) were closely related to the *Bacillus sphaericus* group such as *B. pasteurii*, *B. psychrophilus*, *B. globispourus*, and *Planococcus okeanokoites* (Hammes et al., 2003). Other studies that used ureolytic $CaCO_3$ precipitation for the remediation of divalent cations used *B. pasteurii*, as it is a well known ureolytic microorganism (Fujita et al., 2000; Fujita et al., 2004).

The purpose of this study was to isolate and enrich bacterial isolates with high rates of $CaCO_3$ precipitation. These isolates could then be inoculated into the groundwater to optimize the system for $CaCO_3$ precipitation. The hypothesis for this study is that the inoculation of isolated ureolytic organisms will increase the precipitation of $CaCO_3$ and decrease the dissolved arsenic in both the groundwater and broth media.

4.2 Materials and Methods

4.2.1 Culture selection

Cultures of groundwater bacteria were selected using dilution plating from unfiltered samples taken during the second bioreactor experiment detailed in Chapter 3. Urea test agar was used for the dilution plating and individual cultures were selected and grown in a urea test broth described in detail below with an addition of 15 g L^{-1} of granulated agar (BBL Agar, Becton Dickinson, Oakville, Ont.). Fifteen cultures were isolated on the urea test agar, and grown in urea test broth labeled 1 through 15. Each culture was sampled daily over a period of 7 days and the free Ca(II) was measured using a Ca(II) ISE (3.2.1). The cultures with the largest Ca(II) reduction in solution were then transferred to fresh urea test broth with 5 mM of nitrate added ($NaNO_3$) and grown for 16 d in an anaerobic N_2 atmosphere. Samples from day 0 and day 16 were measured with the Ca(II) ISE to determine which culture was associated with the greatest decrease in Ca(II). Those selected cultures were then chosen for the inoculation experiments.

The selected cultures were spun down in a centrifuge for 10 minutes at 4000 rpm 3 time and washed with sterile groundwater between each spin. After washing the bacterial pellet the culture was resuspended in sterile groundwater to obtain the cell

density to inoculate approximately the same amount into each inoculation treatment. Cell densities were acquired using the Klett-Summerson photoelectric colorimeter. After washing both selected culture pellets with sterile groundwater. Cell densities were obtained using a Klett-Summerson photoelectric colorimeter. Culture 3 had an optical density of 0.076 and culture 4 had a optical density of 0.065, so 1 mL of culture 3 was used for inoculation to 1.2 mL of culture 4. Left over cultures were stored in a -80°C freezer with 50:50 100% glycerol added to preserve bacterial cells.

Urea Test Broth. Urea test broth was made using 0.1 g L^{-1} nutrient broth (DIFCO), 2 mM sodium hydrogen carbonate ($NaHCO_3$) (Assurance BDH, VWR international), 30 mM calcium in the form of anhydrous calcium chloride ($CaCl_2$) (EMD chemicals, Gibbstown, NJ), 5 mM of nitrate for the anaerobic experiments ($NaNO_3$) (Assurance BDH, VWR international) and 0.001 g L^{-1} of phenol red ($C_{19}H_{14}O_5S$) (Fisher Scientific, Ottawa, Ont.). Phenol red is a pH indicator which is yellow below a pH value of 6.8 and turns a pink red colour when the pH increases above 8. The broth was autoclaved for 20 minutes in the liquid setting, and cooled to 50°C in a water bath. The 100 mM chemical grade urea ($(NH_2)_2CO$) (J.T. Baker, Phillipsburg, NJ) was added to half of the broth through filter sterilization using a 0.22 μm syringe filter in a biosafety cabinet. The pH was adjusted to 6.8 (yellow colour) by adding drops of 1 N nitric acid (HNO_3) (Omni*Trace*®).

4.2.2 Inoculation experiments

Groundwater. Groundwater was collected at Riverhurst, Saskatchewan (section 3.2.1).

Broth. The same urea test broth was used as described above in section 4.2.1.

Experiment set up, Duration and Analysis. Two closed culture tube experiments were conducted using both urea test broth and Arkenneth groundwater as the inoculant media. The first culture tube experiment contained no added nitrate while the second experiment contained 5 mM of added nitrate in both the broth and groundwater samples. A total of sixty 50 mL screw-cap Kimax (VWR International) culture tubes were used with 30 tubes containing broth and thirty tubes containing groundwater. In replicates of five, each media had a control and 0.03 M urea treated group either with or without inoculants of cultures 3 and 4. Inoculation of each tube was carried out in a sterile biosafety cabinet to reduce sample contamination.

Broth and groundwater were added to each tube in an N_2 atmosphere to maintain an anaerobic environment. Urea was added via syringe filtration to the treated groups of both the broth and the groundwater media using a sterile 0.22 μm syringe filter in the N_2 atmosphere. Urea was added via pipette to the treated samples every 10 days under a N_2 atmosphere in a glove box using sterile techniques. The broth culture tubes were sampled in a N_2 environment in the glove box after 20 d and again after 35 d for both experiments. The groundwater tubes were only sampled once at the end of the 35 d experiment duration in both experiments.

Calcium and Arsenic Analysis. The 30 broth samples were sampled after 20 d and syringe filtered through a 0.45 μm syringe filter. Free Ca(II) concentration was determined using the Ca^{2+} ISE (3.2.1). The broth samples have a lower ionic strength, and a higher free Ca(II) concentration than the groundwater samples, so a different set of standards had to be made in a broth medium. The Ca(II) ISE was calibrated before, during, and after sample set measurement to account for any electrode drift.

The remaining broth and the groundwater were sampled after 35 d and prepared for arsenic analysis. Hydride generation atomic fluorescence spectroscopy was used to determine arsenic concentrations, (3.2.1). Arsenic concentrations were determined on both the filtered (for dissolved arsenic) and the unfiltered samples (for total arsenic) in both the broth and groundwater samples. Free Ca(II) was determined using the filtered samples for both the broth and groundwater mediums.

Bacterial Viability. The treated inoculated samples of both the broth and groundwater samples of the second experiment were dilution plated at the end of the experiment. A dilution series of 10^{-1}, 10^{-2}, 10^{-4}, 10^{-6}, and finally 10^{-8} were plated out on urea test agar, described above. After 7 d the colony forming units (CFUs) were counted to determine the approximate amount of CFUs from the original sample. This dilution series was simply to verify bacterial viability at the end of the experiment.

Precipitate Analysis. Production of biogenic $CaCO_3$ was verified using several vibrational spectroscopic techniques. The three techniques used were Fourier Transform Infrared (FTIR) microspectrocopy at the Canadian Light Source synchrotron (CLS) using a Bruker Hyperion™ IR microscope (Bruker North America), Fourier Transform Raman (FT-Raman) spectroscopy using a Bruker Optics IFS 66 bench top FT-Raman, and Fourier Transform Photoacustic Infrared (FT-IR-PAS) spectroscopy using a Bruker Optics Vertex 70 bench top FTIR with an MTEC® PAC300 photoacustic detector (Iowa, US). The FTIR and FTIR-PAS techniques utilize light in the mid-IR region (2.5 to 20 micron) and are therefore IR spectroscopy. Raman spectroscopy uses a monochromatic laser at 10^{11} nm wavelength. The photons excite the sample resulting in an energy shift either up or down. IR spectroscopy yields complimentary information, similar to Raman spectroscopy. IR spectroscopy utilizes infrared radiation to determine

the structure of various organic and inorganic compounds. The beam directed at the sample excites the bonds in the structure which may vibrate in different motions (bending or stretching), and the different bond will adsorb the energy at different IR frequencies. Stretching adsorptions produce stronger peaks than bending adsorptions. The different peaks can than be used to identify the chemical structure of the sample. Both IR and Raman spectroscopy are important in the identification of simple molecules because the exclusion rule indicates that if the molecule has a centre of inversion, then it cannot be both Raman and IR active, requiring the use of both techniques (Shriver et al., 1994).

The precipitate formed on the sides of the glass tubes and was removed after the media was sampled using a plastic spatula. The precipitate was dried on silver oxide coated MirrIR™ glass slides (Kevley Technologies, Indianapolis, IN). Calcite standards precipitated by a colleague were used for a $CaCO_3$ reference. FTIR microscopy was used both to obtain detailed images of the biogenic crystals and to collect spectra and confirm the presence of $CaCO_3$. Cultures 3 and 4 were also dried on MirrIR™ glass slides to use as a reference for any bacterial peaks that may be present in the spectra of the broth and groundwater samples.

4.3 Statistical Analysis

Data was analyzed using a two-way general linear model (GLM) using MINITAB 11. Multiple comparisons were performed using least significant difference (LSD). All analysis used a $\alpha=0.05$. The response for the GLM multiple regression was the concentration of the variable being tested, such as the concentration of Ca(II) and arsenic. Factors in the GLM were based on +/- urea, inoculant, and sample preparation

(total and dissolved). For the anaerobic culture selection experiment two way student t-tests, with a 95% confidence level, were used to compare each culture with the control sample. The t-tests were an independent two sample t-test with each data set in a different column comparing the different cultures to the control sample.

4.4 Results

4.4.1 Culture selection experiments

The fifteen cultures isolated from the treated groundwater were grown aerobically in urea test broth. A positive result for urea hydrolysis was defined as a colour change from yellow to red. All fifteen cultures were red in colour after 24 h, and the control broth was slightly pink after 2 d. Each day 5 mL samples were collected in the biosafety cabinet, filtered with a 0.45 μm syringe filter and measured for free Ca(II) concentration (Fig. 4.1). Cultures 3, 4, 5, 6, 7, 8, 9, 10, and 11 were selected to continue with the anaerobic experiments.

The anaerobic selection experiment determined the decrease in free Ca(II) after a period of 16 d. A non-inoculated control was used to determine initial Ca(II) concentrations. All of the eight cultures selected from the previous experiment decreased free Ca(II) significantly compared to the control broth (p<0.01 to 0.001; Fig. 4.2). Cultures 3 and 4 had the overall greatest decrease in Ca(II) from 1387 mg L^{-1} to 841 mg L^{-1} (SD= 49.9) and 773 mg L^{-1} (SD=19.7) respectively.

Cultures 3 and 4 did not have the greatest decrease in Ca(II) in the aerobic selection experiment. However, they did do well anaerobically when nitrate was present. This culture selection process suggests that cultures 3 and 4 could be denitrifiers.

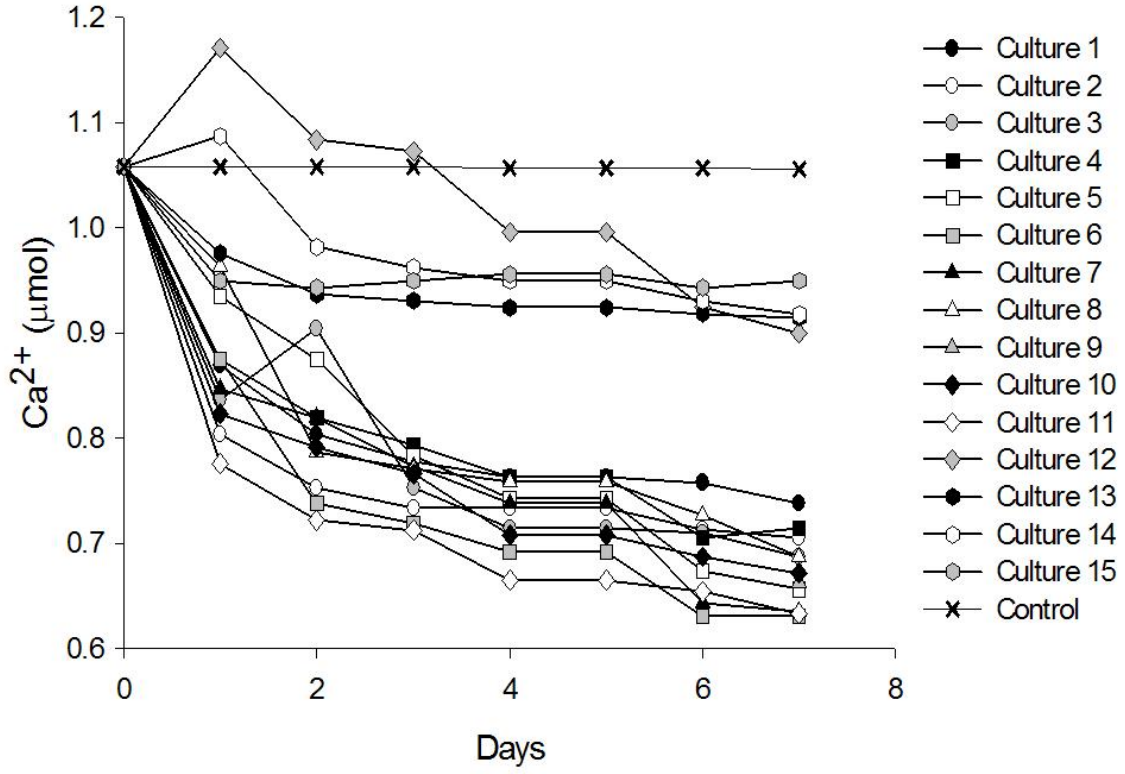

Figure 4.1 Free Ca(II) (µmol) concentrations over the 7 d aerobic culture selection experiment. The fifteen cultures were isolated from Ardkenneth groundwater on urea test agar, transferred to urea test broth and grown aerobically for 7 days.

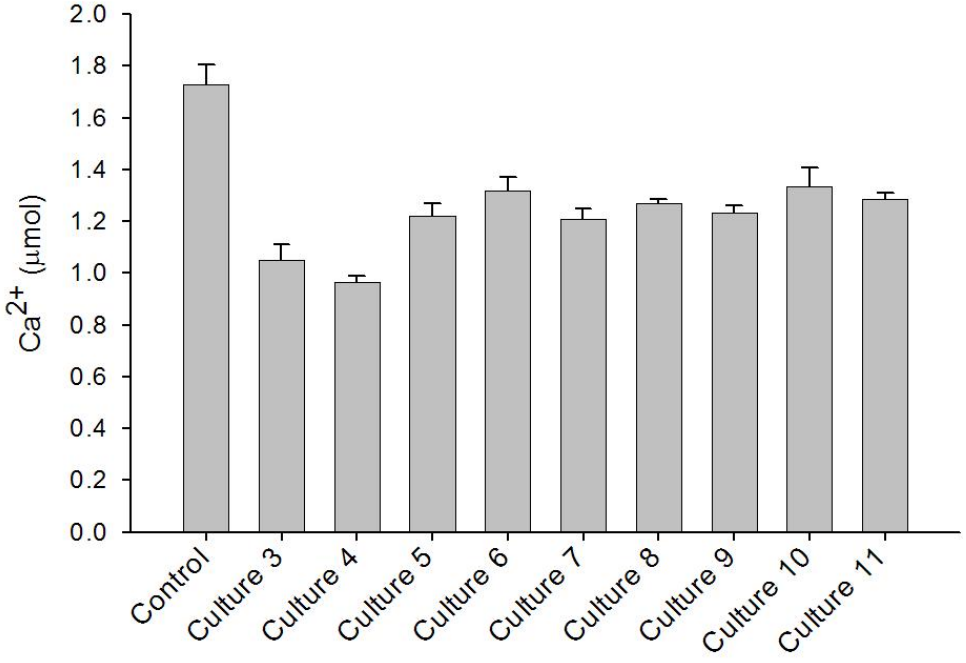

Figure 4.2 Mean free Ca(II) (µmol) for the anaerobic inoculant selection experiment. Cultures were selected from the 7 day aerobic experiment. Cultures were transferred to urea test broth in an anaerobic environment and grown for 16 days. Error bars represent standard deviation for each culture data set.

4.4.2 Inoculation experiments

4.4.2.1 Experiment #1: No added nitrate

The first inoculation experiment was under anaerobic conditions. However, 5 mM nitrate ($NaNO_3$) was not present in either the broth or groundwater media. This first experiment with no nitrate present had negligible precipitate formed in any of the media or treatments. After 20 d the free Ca(II) concentration in the broth was lower in the urea treated group than in the control group ($p<0.01$; Fig. 4.3). There was no difference in Ca(II) concentration between the inoculants used within the control group ($p=0.92$). However, there was a slight difference in Ca(II) concentration between the inoculants and non inoculated samples within the urea treated group because the non inoculant was 0.45 μmol (SD 0.02) and treated broth with cultures 3 and 4 were 0.42 μmol (SD=0.002) and 0.43 μmol (SD=0.005) respectively ($p=0.042$).

After 35 days Ca(II) concentration in the broth was similar to that of the 20 d samples. The urea treated group had a lower free Ca(II) concentration than the control group ($p<0.01$; Fig. 4.4). There was no difference between inoculants within the control treatment; all had Ca(II) concentrations of 0.59 μmol (SD=0.16) ($p=0.972$).

The groundwater samples at the end of the 35 d experiment had lower free Ca(II) in the urea treated group than in the control group ($p<0.01$; Fig. 4.5). There also was a difference in inoculants between the treatments ($p=0.002$) and within the urea treated samples. In the urea treated samples, the non inoculated group had higher free Ca(II) at 0.36 μ mol (SD=0.05) where the culture 3 and 4 samples had 0.34 μ mol (SD=0.06) and 0.37 μ mol (SD=0.02) respectively ($p<0.01$).

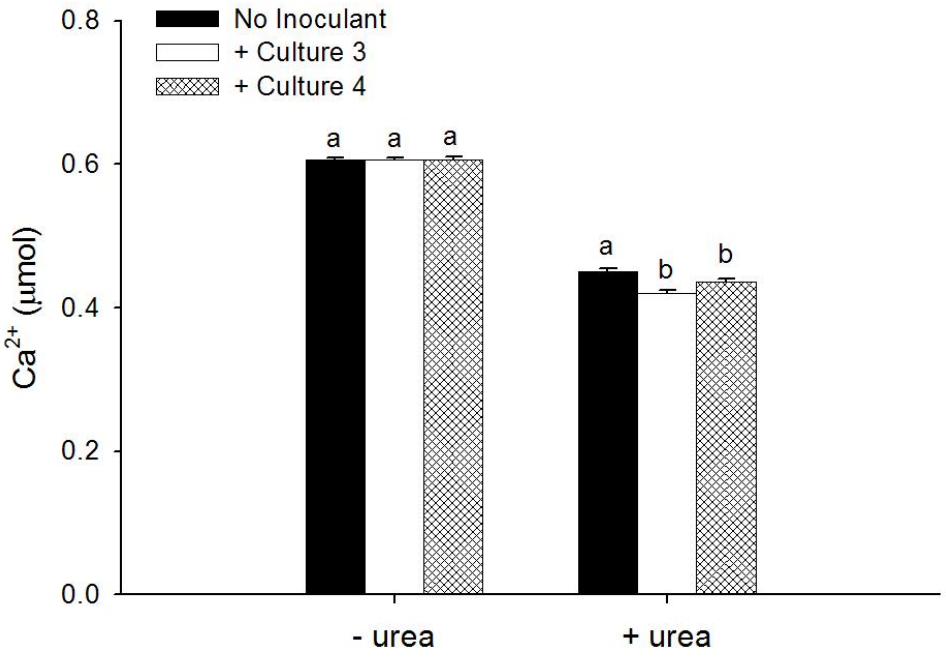

Figure 4.3 Mean free Ca(II) (µmol) in the broth media after 20 d for the first inoculated experiment with no added nitrate. All treatments were kept in an anaerobic environment. Each experimental group of +urea and –urea had three inoculant treatments; control, + culture 3 (1 mL), and + culture 4 (1.2 mL). All treatments had initial arsenic concentrations of 0.07 µg L^{-1} and Ca(II) concentrations of 1300 mg L^{-1}. Bars with the same letter are not significantly different according to $LSD_{0.05}$. Error bars represent the standard deviations (n=5).
† Means of –urea and +urea are significantly different.

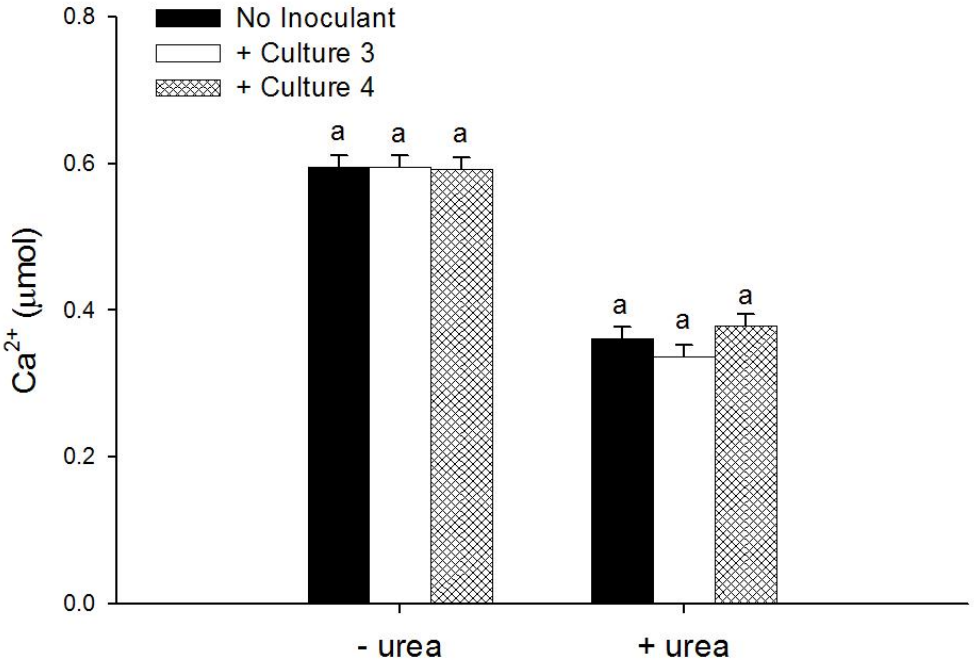

Figure 4.4 Mean free Ca(II) (µmol) in the broth media after 35 d for the first inoculated experiment with no added nitrate. All treatments were kept in an anaerobic environment. Each experimental group of +urea and –urea had three inoculant treatments; control, + culture 3 (1 mL), and + culture 4 (1.2 mL). All treatments had initial arsenic concentrations of 0.07 µg L^{-1} and Ca(II) concentrations of 1300 mg L^{-1}. Bars with the same letter are not significantly different according to $LSD_{0.05}$. Error bars represent the standard deviations (n=5).
† Means of –urea and +urea are significantly different

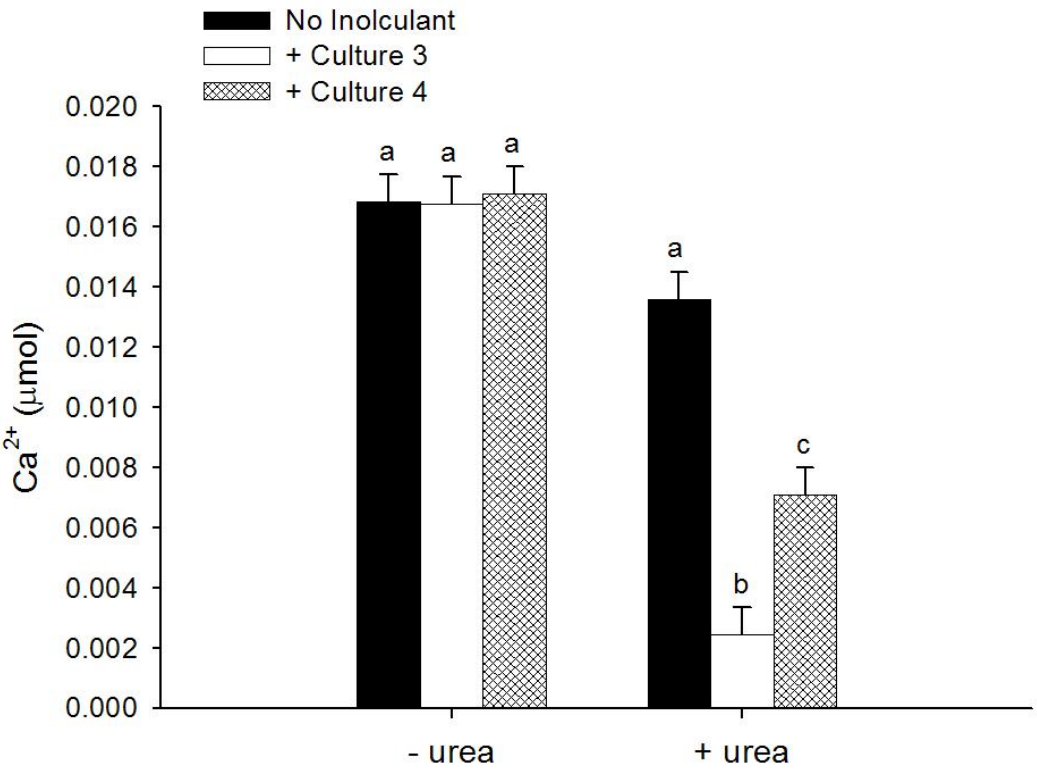

Figure 4.5 Mean free Ca(II) (µmol) in the groundwater media after 35 d for the first inoculated experiment with no added nitrate. All treatments were kept in an anaerobic environment. Each experimental group of +urea and –urea had three inoculant treatments; control, + culture 3 (1 mL), and + culture 4 (1.2 mL). All treatments had initial arsenic concentrations of 0.07 µg L^{-1} and Ca(II) concentrations of 36 mg L^{-1}. Bars with the same letter are not significantly different according to $LSD_{0.05}$. Error bars represent the standard deviations (n=5).
†Means of –urea and +urea are significantly different

Arsenic data was collected after the 35 d duration of the experiment. Both filtered and unfiltered samples were analyzed to obtain dissolved and total arsenic data for the experiment, respectively. There was no difference in broth sample preparation for dissolved and total arsenic (p=0.079). Thus, particulate arsenic was not calculated (Fig. 4.6). The arsenic was much lower in the treated group at 0.1 μ mol (SD=0.1) when compared to the control group at 0.52 μ mol (SD=0.05) (p<0.01). However, there was no difference between the inoculations within each treatment (p=0.465).

The arsenic data in the groundwater samples differed from the broth samples in that there was no difference between the untreated control and urea treated groups (p=0.149). Unlike the broth samples, there was a difference between the total and dissolved arsenic in the groundwater samples because of substantial particulate arsenic. The total arsenic samples had a much higher concentration (about 0.15 μ mol) when compared to the dissolved arsenic at 0.022 μ mol (p<0.01) and thus particulate arsenic was calculated (Fig. 4.7). Similar to the broth samples the groundwater samples had no difference between the inoculations within each treatment in the dissolved arsenic (p=0.761). There was a small difference in arsenic concentrations between the urea treatments in the particulate samples (p=0.047). There was no difference in arsenic concentrations between the inoculants in the urea treated group in the particulate arsenic samples (p=0.085; Fig.4.7).

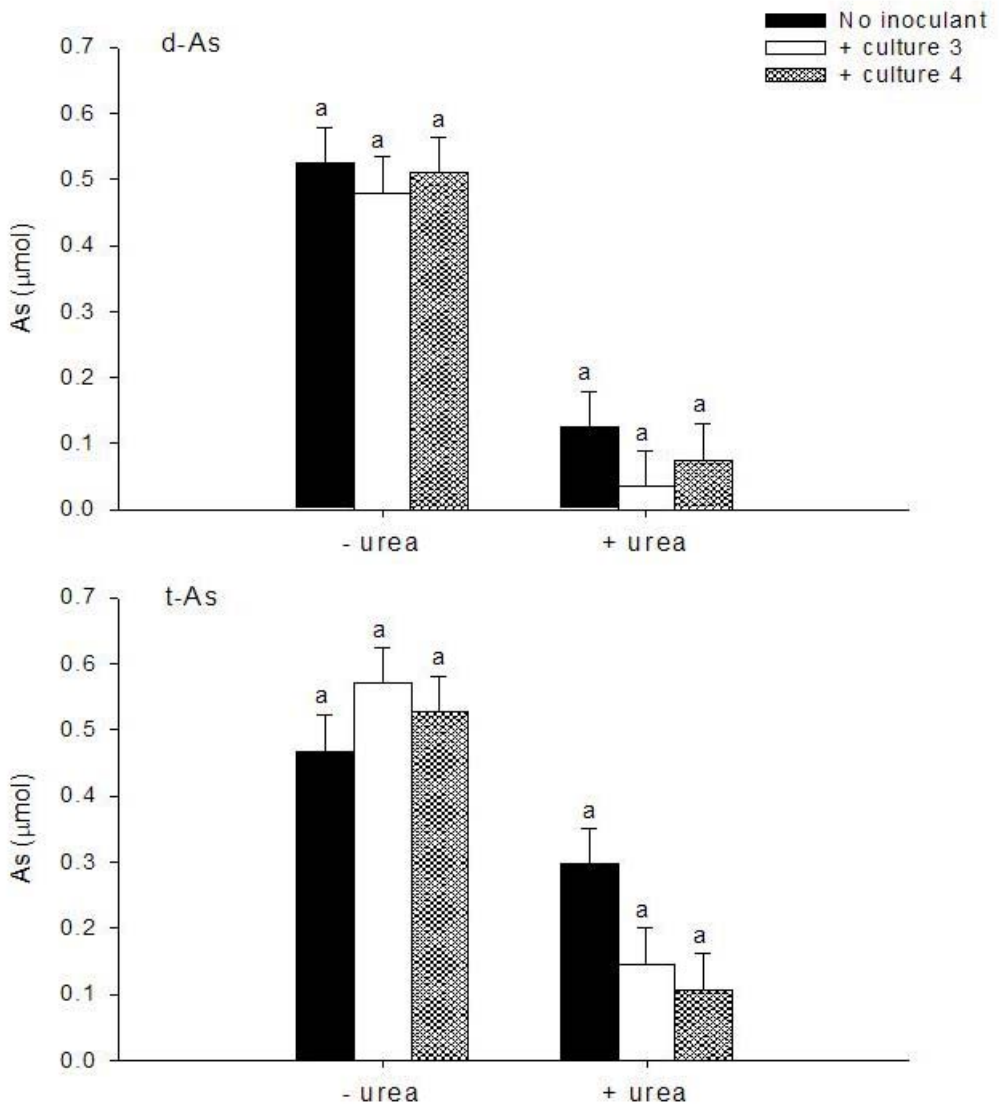

Figure 4.6 Mean dissolved arsenic (d-As) and total arsenic (t-As) concentrations (µmol) for the broth samples after 35 d of the first inoculation experiment with no added nitrate. All treatments were kept in an anaerobic environment. Each experimental group of +urea and –urea had three inoculant treatments; control, + culture 3 (1 mL), and + culture 4 (1.2 mL). All treatments had initial arsenic concentrations of 0.07 µg L^{-1} and Ca(II) concentrations of 1300 mg L^{-1}. Bars with the same letter are not significantly different according to $LSD_{0.05}$. Error bars represent the standard deviations (n=5).

†Means of –urea and +urea for both d-As and t-As are significantly different

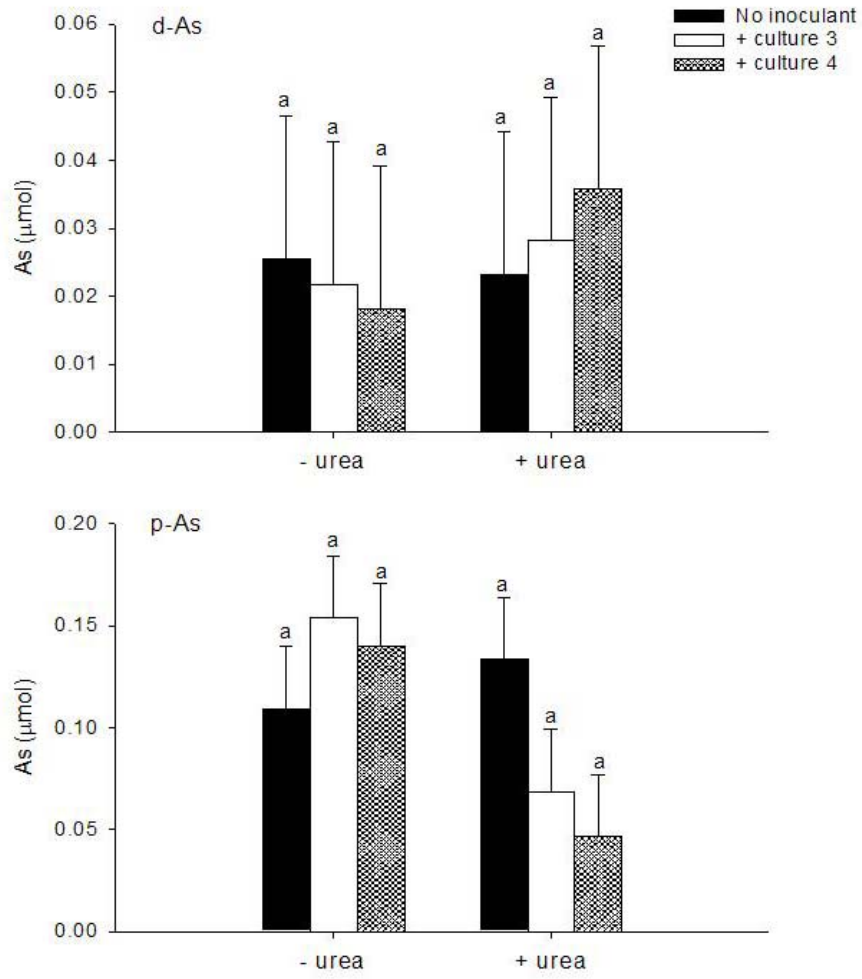

Figure 4.7 Mean dissolved arsenic (d-As) and particulate arsenic (p-As) concentrations (µmol) for the groundwater samples after 35 d of the first inoculation experiment with no added nitrate. All treatments were kept in an anaerobic environment. Each experimental group of +urea and –urea had three inoculant treatments; control, + culture 3 (1 mL), and + culture 4 (1.2 mL). All treatments had initial arsenic concentrations of 0.07 µg L^{-1} and Ca(II) concentrations of 36 mg L^{-1}.Bars with the same letter are not significantly different according to $LSD_{0.05}$. Error bars represent the standard deviations (n=5).

†Means if –urea and +urea for the d-As are not significantly different where as means for the urea and +urea for the p-As are significantly different.

4.4.2.2 Experiment #2: Nitrate added

The second experiment included 5 mM nitrate in both the broth and groundwater samples, and was much more successful than the first experiment in forming a precipitate. After 20 days free Ca(II) was measured using an ISE and it was found that the control was much higher than the +urea treatment group for all inoculants (Fig. 4.8; $p<0.01$). Within the –urea control group there was no difference in Ca(II) concentration between the treatments ($p=0.088$). Within the +urea treated group, the non inoculated samples had a higher Ca(II) concentration at 0.48 µ mol when compared to both culture 3 and 4 at 0.382 µ mol (SD=0.007) and 0.383 µ mol (SD=0.028) ($p=0.0078$ and $p=0.0057$). There was no difference between the two inoculants within the urea treated group ($p=0.92$).

At the end of the 35 d experiment the free Ca(II) for the broth samples was similar to that after 20 d in that the control was higher in Ca(II) concentration (µmol) than the treated group ($p<0.01$). Also similar to the data after 20 d the free Ca(II) after 35 d had no difference between the inoculants within the control group at 0.54 µ mol (SD=0.015) ($p=0.366$). The inoculants did differ within the treated group ($p<0.01$), as the non inoculated sample with 0.5 µ mol (SD=0.04) Ca(II) was much higher than both inoculated samples with only 0.24 µ mol (SD=0.009) (Fig. 4.9).

Similar to the broth samples the groundwater samples had higher free Ca(II) in the control samples ($p<0.01$; Fig. 4.10). There was also a difference between inoculated treatments within the treated group ($p=0.008$). The two inoculated treatments had lower free Ca(II) than the non inoculated group.

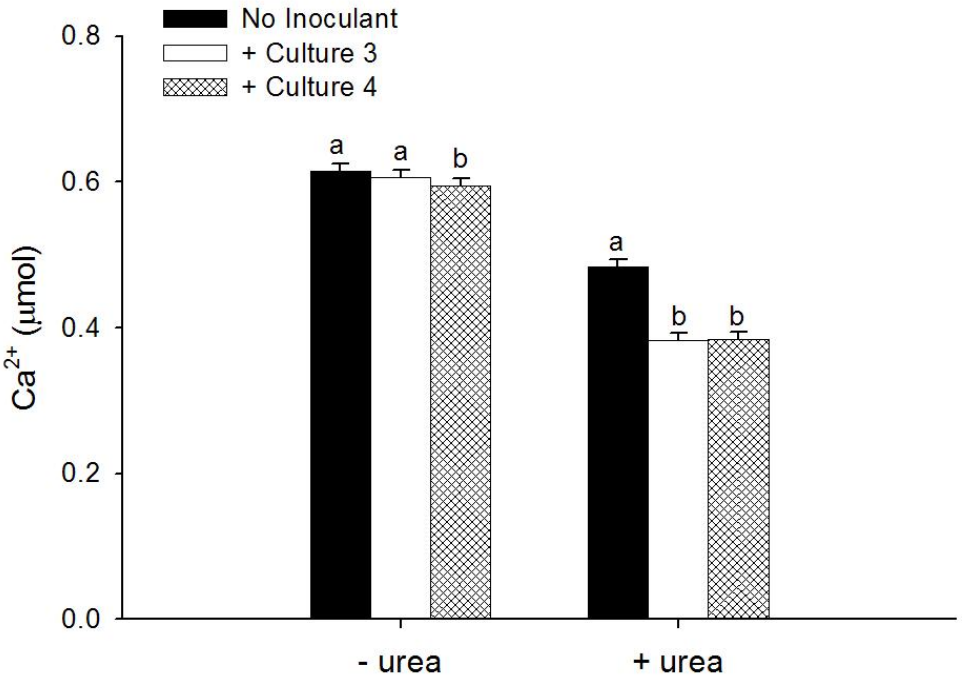

Figure 4.8 Mean free Ca(II) (µmol) in the broth media after 20 d for the second inoculated experiment with added nitrate. All treatments had 5 m*M* nitrate added and were kept in an anaerobic environment. Each experimental group of +urea and –urea had three inoculant treatments; control, + culture 3 (1 mL), and + culture 4 (1.2 mL). All treatments had initial arsenic concentrations of 0.07 µg L^{-1} and Ca(II) concentrations of 1300 mg L^{-1}. Bars with the same letter are not significantly different according to $LSD_{0.05}$. Error bars represent the standard deviations (n=5).

†Means of –urea and +urea are significantly different

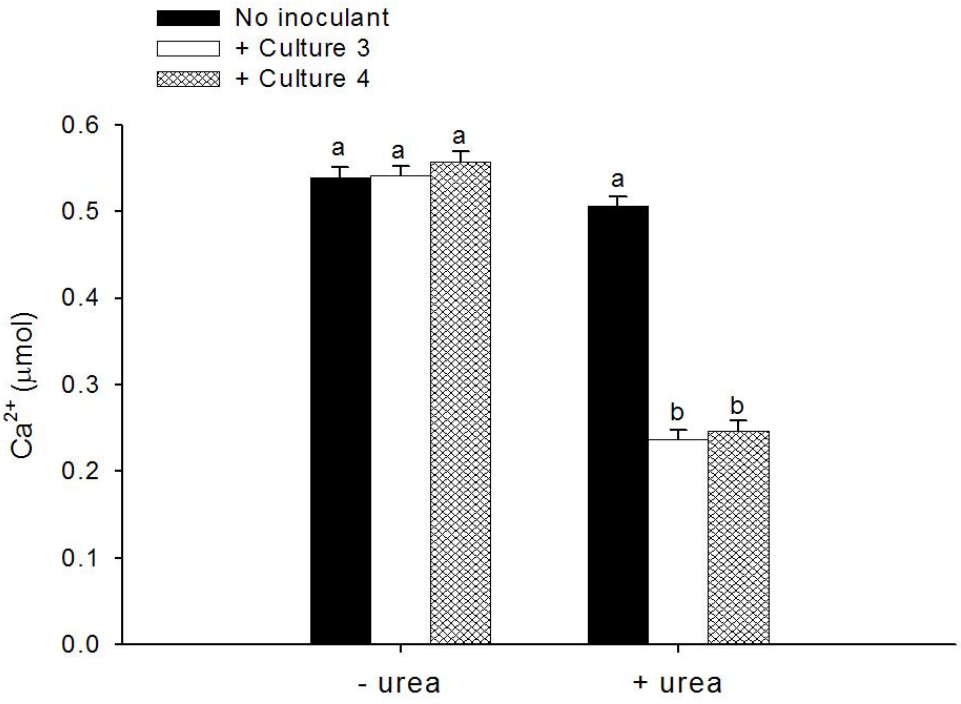

Figure 4.9 Mean free Ca(II) (µmol) in the broth media after 35 d for the second inoculated experiment with added nitrate. All treatments had 5 m*M* nitrate added and were kept in an anaerobic environment. Each experimental group of +urea and –urea had three inoculant treatments; control, + culture 3 (1 mL), and + culture 4 (1.2 mL). All treatments had initial arsenic concentrations of 0.07 µ L^{-1} and Ca(II) concentrations of 1300 mg L^{-1}. Bars with the same letter are not significantly different according to $LSD_{0.05}$. Error bars represent the standard deviations.

†Means of –urea and +urea are significantly different

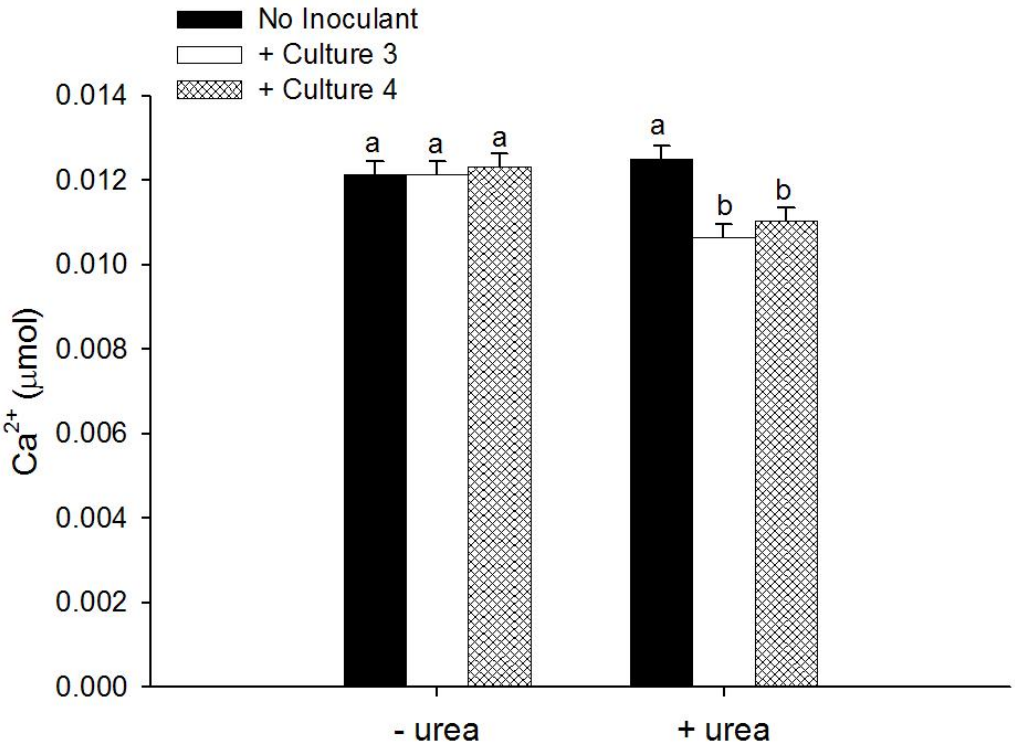

Figure 4.10 Mean free Ca(II) (µmol) in the groundwater media after 35 d for the second inoculated experiment with added nitrate. All treatments had 5 m*M* nitrate added and were kept in an anaerobic environment. Each experimental group of +urea and –urea had three inoculant treatments; control, + culture 3 (1 mL), and + culture 4 (1.2 mL). All treatments had initial arsenic concentrations of 0.07 µg L^{-1} and Ca(II) concentrations of 36 mg L^{-1}. Bars with the same letter are not significantly different according to $LSD_{0.05}$. Error bars represent the standard deviations.

†Means of –urea and +urea are significantly different

Arsenic concentrations were collected at the end of the 35 d experiment for both total and dissolved arsenic samples. The total arsenic concentrations were greater than the dissolved arsenic concentrations ($p<0.01$), thus particulate arsenic was determined (Fig. 4.11). The control group had greater arsenic concentrations that the treated group independently of the inoculants ($p<0.01$). Inoculation of cultures 3 and 4 in the urea treated group did lower arsenic to concentrations of 0.26 µmol (SD=0.21) and 0.47 µmol (SD=0.25) ($p=0.028$) when compared to the non inoculated concentration of 0.59 µmol (SD=0.5) (Fig. 4.11). The concentrations for particulate arsenic were higher in the control group that the 0.03 *M* urea treated group ($p=0.039$). There were no differences between the inoculants within the 0.03 *M* urea treated group ($p=0.565$). In the control group there was no difference between non inoculated and culture 4 ($p=0.123$). However, culture 3 had lower particulate arsenic ($p=0.04$) than the non inoculated and culture 4 (Fig. 4.11).

The arsenic concentrations from the groundwater samples were similar to the broth samples in that the total arsenic concentrations were greater than the dissolved arsenic concentrations ($p=<0.001$). Particulate arsenic concentrations were calculated from the total and dissolved arsenic data (Fig. 4.12). Unlike the broth samples, there was no difference in arsenic concentration within the untreated and urea treated samples, independent of the sample inoculation ($p=0.10$). Also, unlike the broth samples there was no difference between the inoculants within each treatment group of the dissolved arsenic concentrations ($p=0.212$). There were also no differences between the inoculants in both the untreated control and urea treated groups of the particulate arsenic ($p=0.06$ and $p=0.369$).

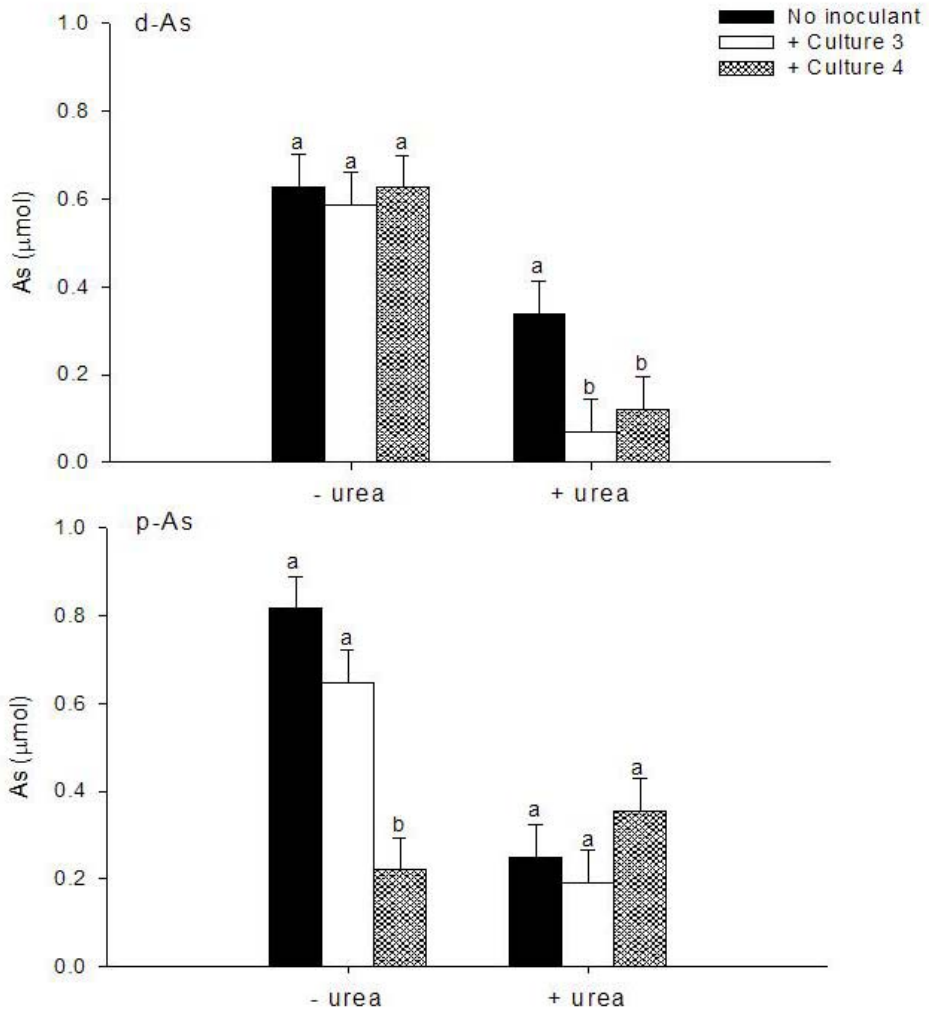

Figure 4.11 Mean dissolved arsenic (d-As) and particulate arsenic (p-As) concentrations (µmol) for the broth samples after 35 d of the second inoculation experiment with added nitrate. All treatments had 5 m*M* nitrate added and were kept in an anaerobic environment. Each experimental group of +urea and –urea had three inoculant treatments; control, + culture 3 (1 mL), and + culture 4 (1.2 mL). All treatments had initial arsenic concentrations of 0.07 µg L^{-1} and Ca(II) concentrations of 1300 mg L^{-1}. Bars with the same letter are not significantly different according to $LSD_{0.05}$. Error bars represent the standard deviations (n=5).
†Means of –urea and +urea are significantly different

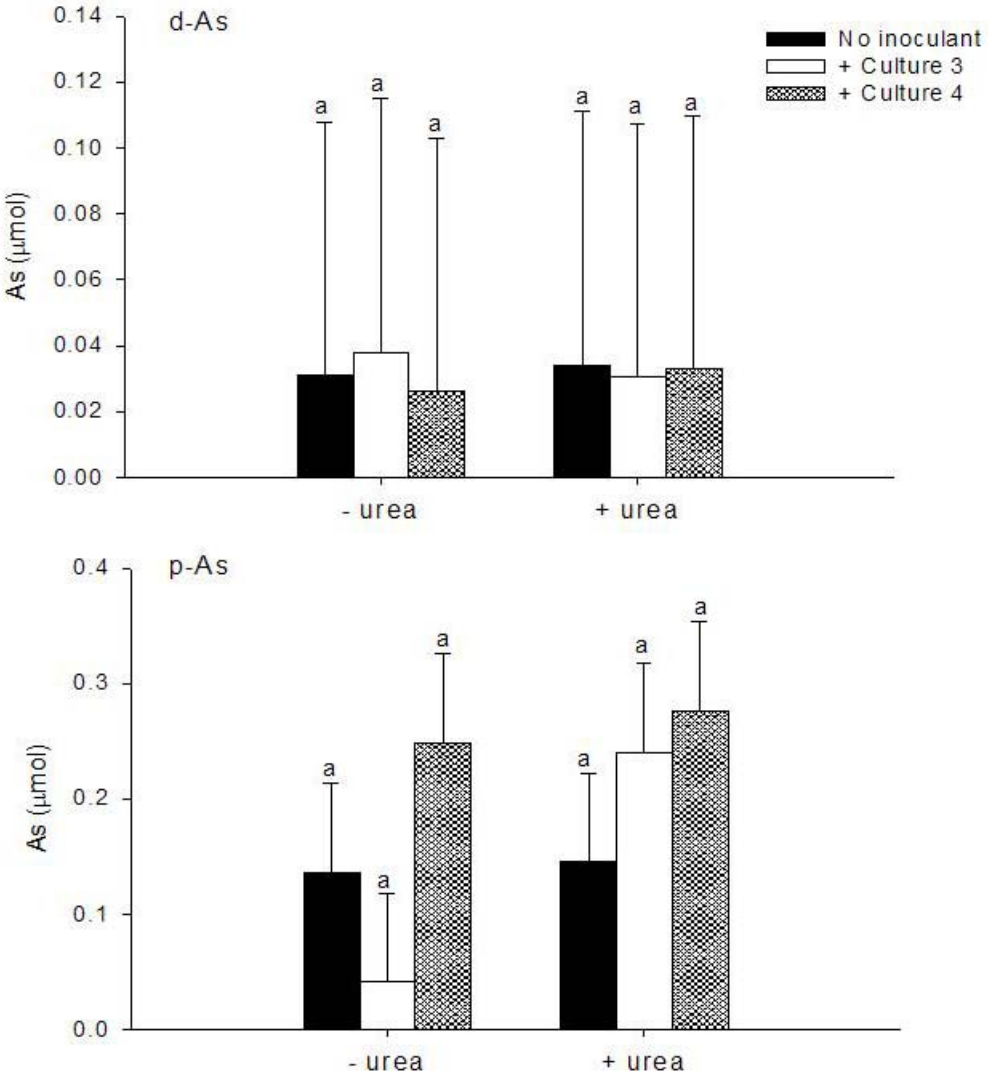

Figure 4.12 Mean dissolved arsenic (d-As) and particulate arsenic (p-As) concentrations (µmol) for the groundwater samples after 35 d of the second inoculation experiment with added nitrate. All treatments had 5 m*M* nitrate added and were kept in an anaerobic environment. Each experimental group of +urea and –urea had three inoculant treatments; control, + culture 3 (1 mL), and + culture 4 (1.2 mL). All treatments had initial arsenic concentrations of 0.07 µg L^{-1} and Ca(II) concentrations of 36 mg L^{-1}. Bars with the same letter are not significantly different according to $LSD_{0.05}$. Error bars represent the standard deviations (n=5).
†Means of –urea and +urea for both d-As and p-As are not significantly different

Bacterial viability at the end of the second experiment was ensured using dilution plating to determine CFUs. Inoculated cultures survived in the urea treated group of the broth and groundwater media (Fig. 4.13). There was a large difference between the broth and groundwater CFU values for both inoculations. The broth CFU L^{-1} was much greater than the CFU L^{-1} for the groundwater samples ($p<0.01$). There was no difference between culture 3 and 4 in the broth or the groundwater samples ($p=0.549$ and $p=0.366$).

The precipitate formed in the inoculated urea treated samples from both media was analyzed using different IR spectroscopy techniques. Both broth samples produced a large amount of precipitate and the crystals were amorphous in shape observed with the IR microscope. The groundwater inoculated samples also had amorphous shaped crystals. IR microscopy allows for images of the samples before using the FTIR to identify the samples. FTIR microscopy of both pure cultures show characteristic peaks at 2963 cm^{-1}, 2929 cm^{-1}, and 2972 cm^{-1} which represent the CH_3 and CH_2 groups in fatty acids in Fig. 4.14 (Table 4.1) (Naumann, 2000). Culture 3 had higher peaks at 1738 cm^{-1}, 1661 cm^{-1}, and 1547 cm^{-1} representing the esters in DNA and RNA, and Amide I and II bands respectively (Naumann, 2000). Culture 3 also had higher peaks in the CH_2 and COO^- region of 1453 cm^{-1} and 1401 cm^{-1}, as well as amide III bands at 1302 cm^{-1} and 1246 cm^{-1} (Table 4.1) (Naumann, 2000). Crystals in the 0.03 *M* urea treated groundwater samples inoculated with culture 3 and 4 are sub-angular to amorphous in shape, surrounded by a matrix likely composed of salts from the groundwater (Fig. 4.15). The spectra from each of the two crystals in each image (Fig. 4.15) were averaged to make one spectra for each sample (Fig. 4.16).

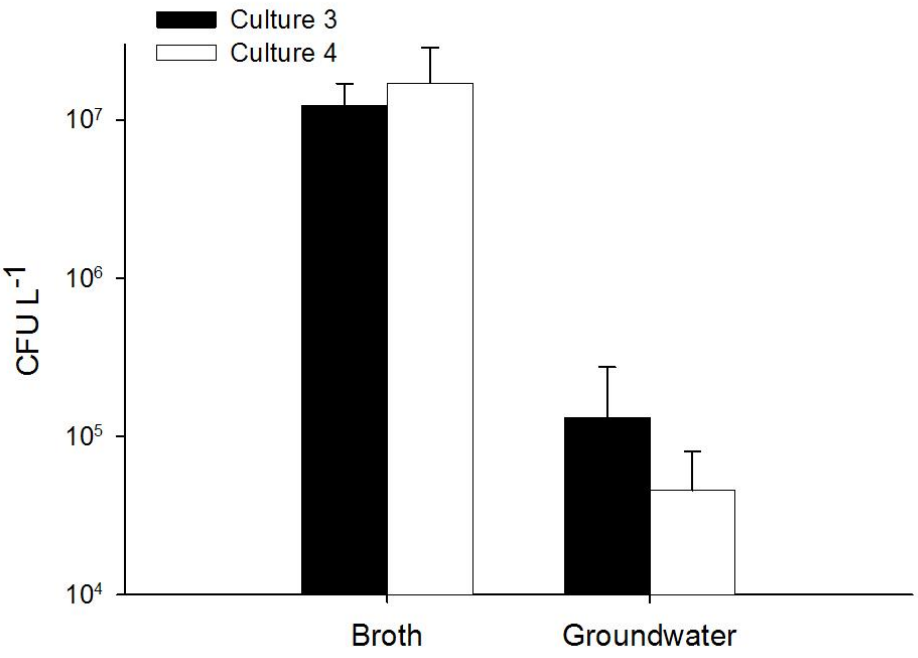

Figure 4.13 Average colony forming units (CFU L^{-1}) for the inoculated urea treated broth and groundwater samples. Error bars represent the standard deviations.

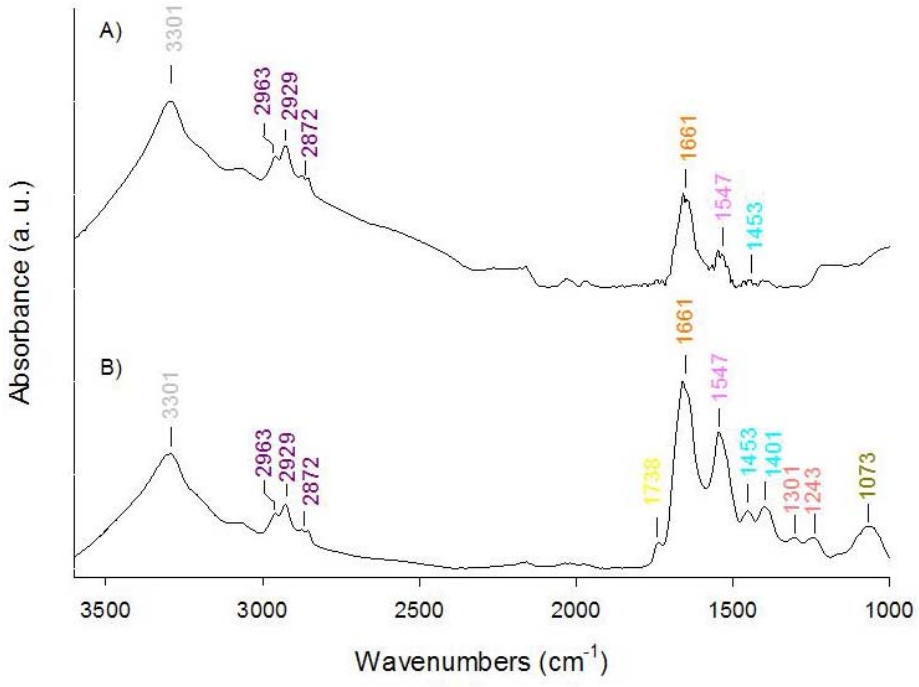

Figure 4.14 Fourier Transform Infrared (FTIR) microscopy spectra of A) culture 4 and B) culture 3. Pure cultures were dried on MirrIR glass slides and analyzed using FTIR microscopy. The marked peaks represent different key components in a bacterial cell, such as the fatty acids (2963 cm^{-2} to 2872 cm^{-1}), the amide bands I II and III (1661 cm^{-1} to 1547 cm^{-1} and 1301 cm^{-1} to 1243 cm^{-1}), and carbohydrates (1077 cm^{-1}).

Table 4.1 Wavenumber (cm^{-1}) assignments for bands found in the bacterial cultures using IR microscopy (Naumann, 2000). Colours have been assigned to each component.

Band Assignment	Wavenumber cm^{-1}
Hydroxyl group (O-H stretch)	~ 3301
	~ 2963
Fatty acids (C-H stretch)	~ 2929
	~ 2972
DNA/RNA (C=O esters)	~ 1738
Amide I	~ 1661
Amide II	~ 1547
Amide III	~ 1301, 1243
Amino acids (C=O and C-H stretch)	~ 1453, 1401
Carbohydrates (C-O-C, C-O)	~ 1073

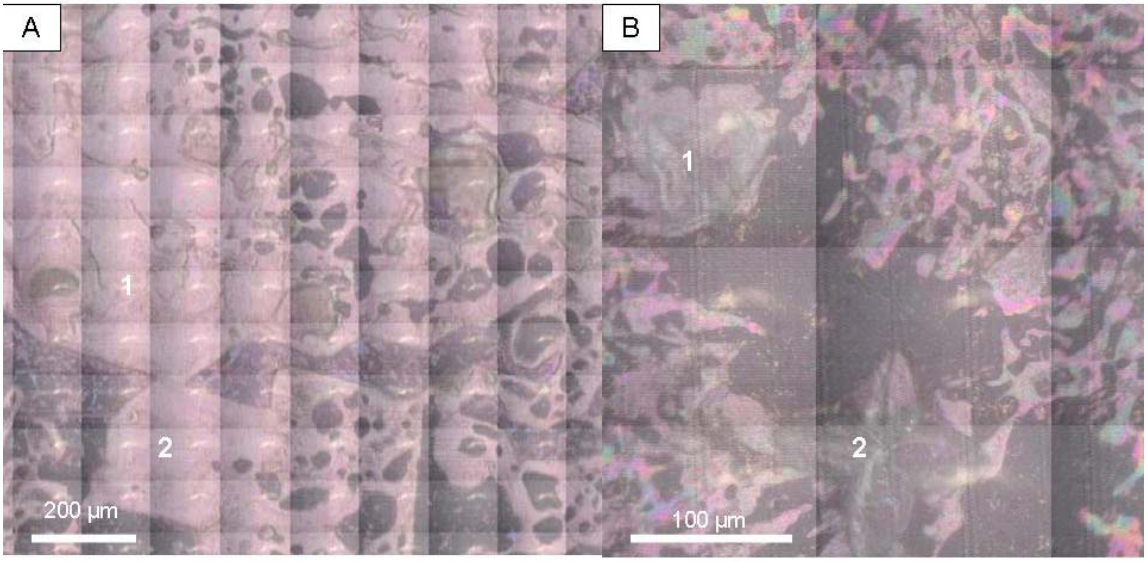

Figure 4.15 FTIR microscope images of the 0.03 *M* urea treated groundwater samples A) inoculated with culture 3 and B) inoculated with culture 4. The FTIR spectra are an average of spectra from the two crystals in each image.

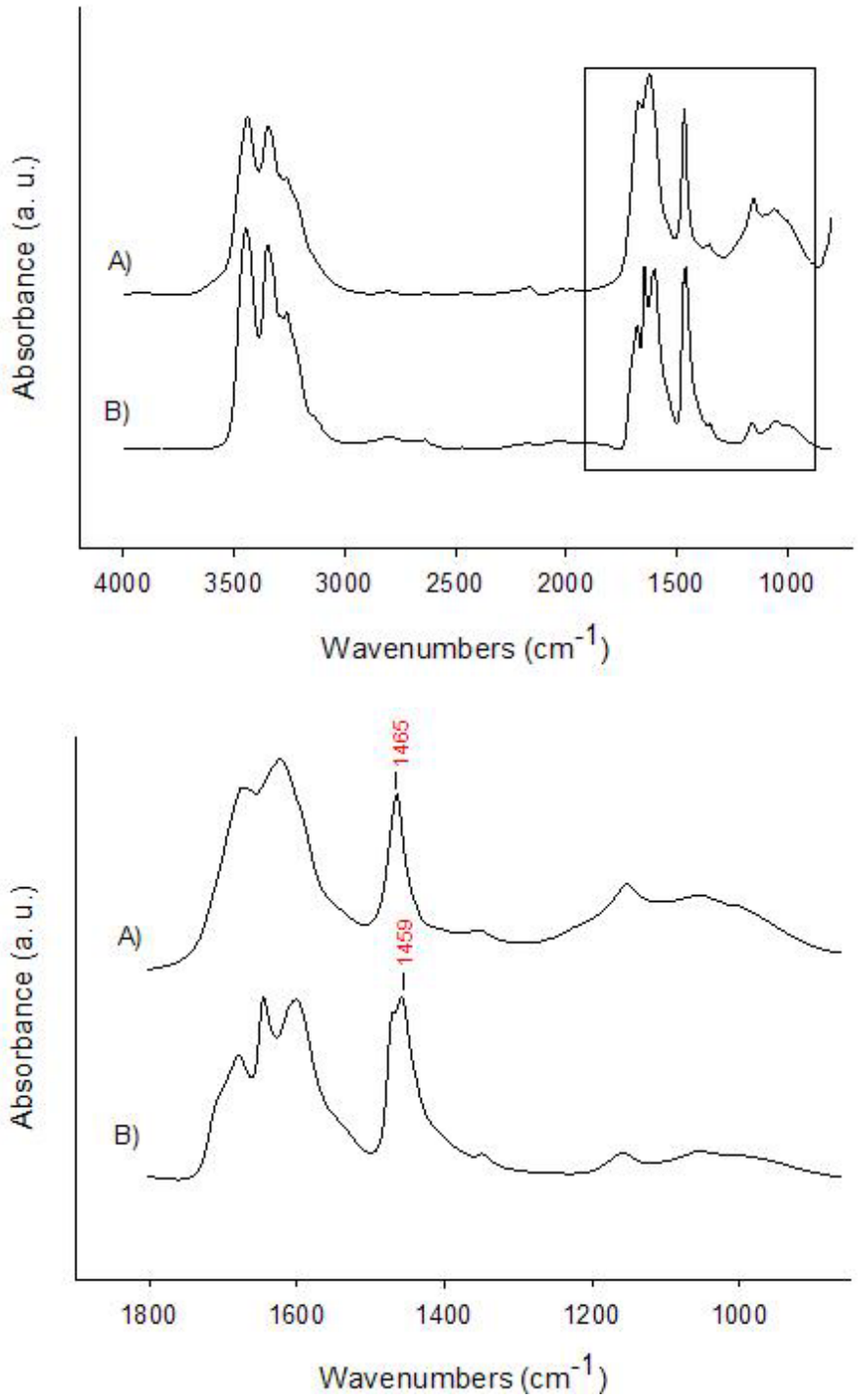

Figure 4.16 Fourier Transform Infrared (FTIR) microscopy spectra of A) urea treated groundwater inoculated with culture 4, and B) urea treated groundwater inoculated with culture 3. The marked peaks at 1459 cm^{-1} and 1465 cm^{-1} represent the presence of carbonate (CO_3) in the sample.

FTIR microscopy of the 0.03 *M* urea treated groundwater samples show characteristic peaks for carbonate (CO_3) at 1459 cm^{-1} in sample inoculated with culture 3 and 1463 cm^{-1} in the samples inoculated with culture 4 (Fig. 4.16). The 0.03 *M* urea treated broth samples had amorphous crystals that appear out of focus with the FTIR microscope (Fig. 4.17). FTIR microscopy of the urea treated broth samples have similar carbonate peaks at 1464 cm^{-1}, where as the culture 4 sample has an amide I band at 1642 cm^{-1} and fatty acid peaks at 2875 cm^{-1} and 2933 cm^{-1} (Fig. 4.18).

The broth spectra in Fig. 4.18 show characteristic peak for both calcite and aragonite at 873 cm^{-1} and 860 cm^{-1}. This band split in the 875 cm^{-1} to 855 cm^{-1} range could indicate that a mixture of calcite and aragonite has been formed by urea hydrolysis. The peak at 1796 cm^{-1} could be a carbonyl group (C=O) with associated electronegative groups close by (Colthup et al., 1990).

Two other IR spectroscopy techniques were used to verify the presence of $CaCO_3$ in the urea treated samples (FT-Raman and FTIR-PAS). The FT-Raman spectroscopy compared the urea treated broth and groundwater samples with chemically precipitated calcite by a colleague. The results of the FTIR-Raman and FTIR-PAS are summarized in Table 4.2). The all samples had the same Raman active peaks indicating calcite at 1086 cm^{-1} and 715 cm^{-1} (Fig. 4.19). The urea treated groundwater samples had Raman active peaks at 1067 cm^{-1} and 724 cm^{-1}. These Raman active peaks indicate the presence of NO_3 in the system (Colthup et al., 1990; Nakamoto, 1970). Also the groundwater samples have gypsum ($CaSO_4$) indicated by bands at 1177 cm^{-1} and 1012 cm^{-1} (Fig. 4.19) (Table 4.2) (Farmer, 1974). The gypsum would have precipitated out as a salt when the groundwater samples dried as free Ca(II) remained in solution after the precipitation of small amounts of $CaCO_3$.

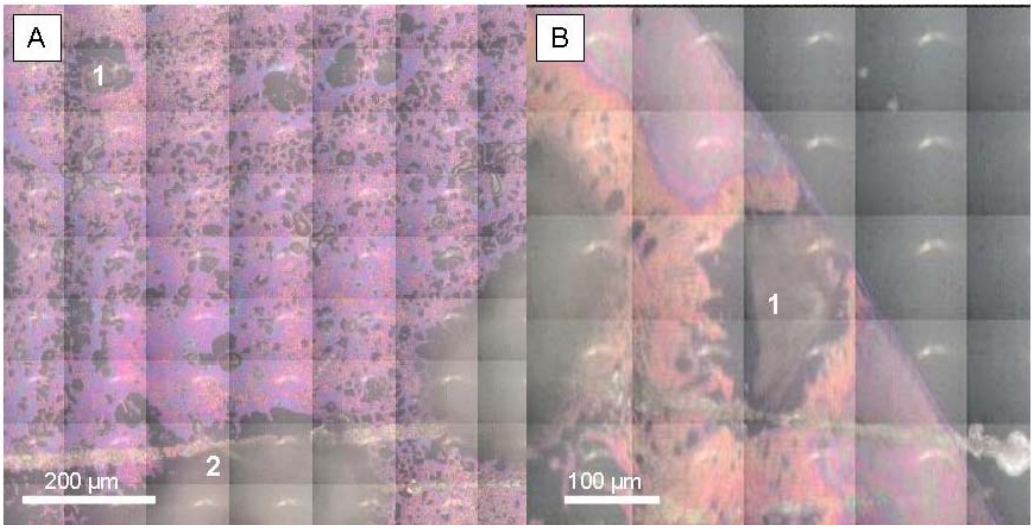

Figure 4.17 FTIR microscope images of the 0.03 *M* urea treated broth media samples A) inoculated with culture 3 and B) inoculated with culture 4. The FTIR spectra are an average of spectra from the two crystals in the culture 3 image and an average of the many points on the crystal in the culture 4 image.

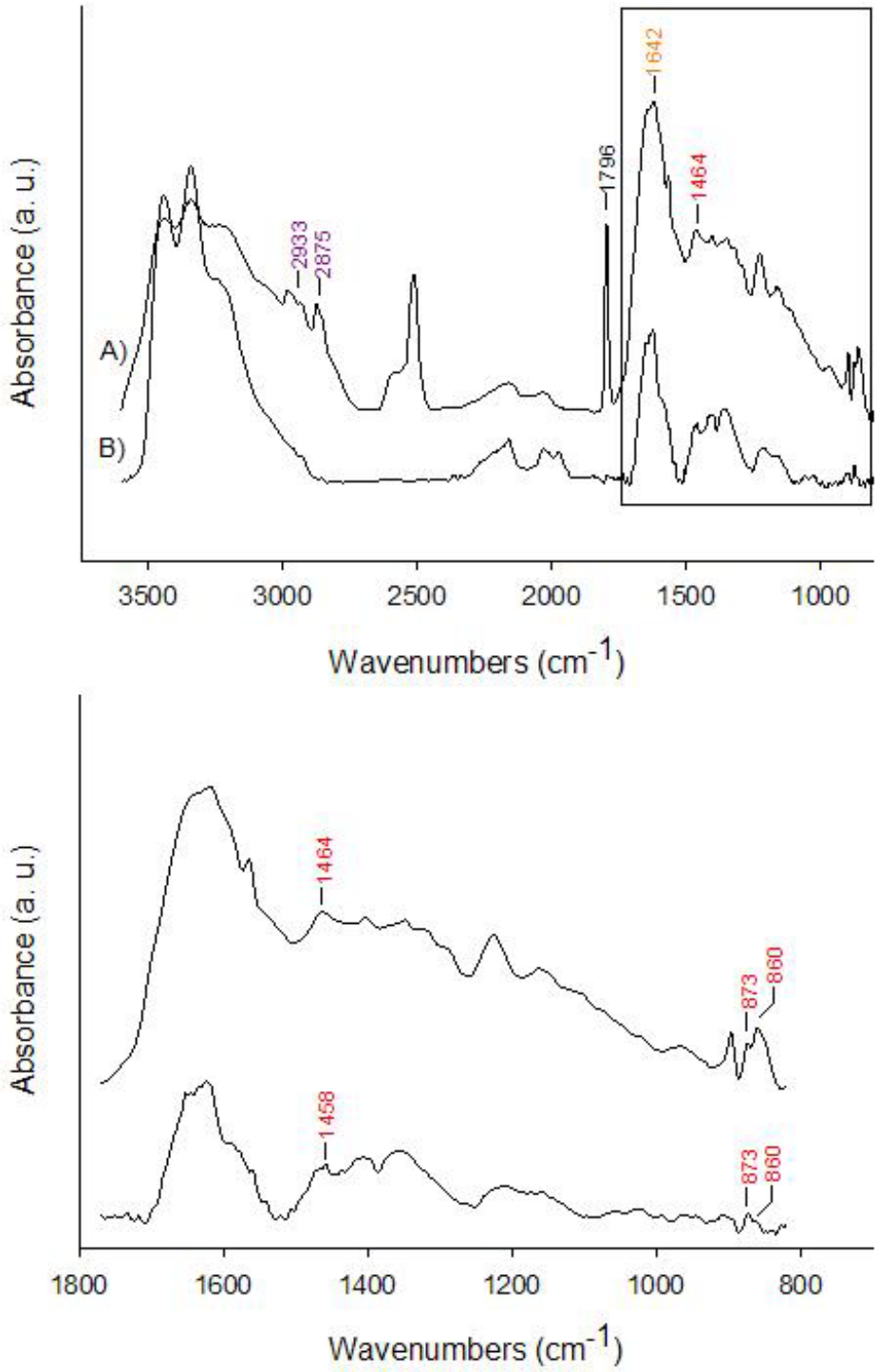

Figure 4.18 Fourier Transform Infrared (FTIR) microscopy spectra of A) urea treated broth inoculated with culture, 4 and B) urea treated broth inoculated with culture 3. The marked peaks in both samples represent the presence of carbonate (1464 cm^{-1}) and the amide I band (1642 cm^{-1}). Spectra A) also has characteristic peaks for the fatty acids in a bacterial culture (2875 cm^{-1} and 2933 cm^{-1}). The peaks at 873 cm^{-1} and 860 cm^{-1} are characteristic of calcite and aragonite peaks.

Table 4.2 Summery of wavenumber (cm^{-1}) assignments for the bands found in the FTIR and FTIR-Raman techniques for identification of inorganic salts. Colours have been assigned to each component.

Band Assignment	Vibrational mode and Wavenumber (cm^{-1})			
	ν_1	ν_2	ν_3	ν_3
$CaCO_3$	1086†	876, 848‡	1450-1460	715†
$CaSO_4$	1012†	420§	1177†	623§
$NaNO_3$	1067†			724†

† Raman active bands
‡ Band split indicative of a calcite aragonite mixture
§ Bands not observed in this study (Sarma et al., 1998)

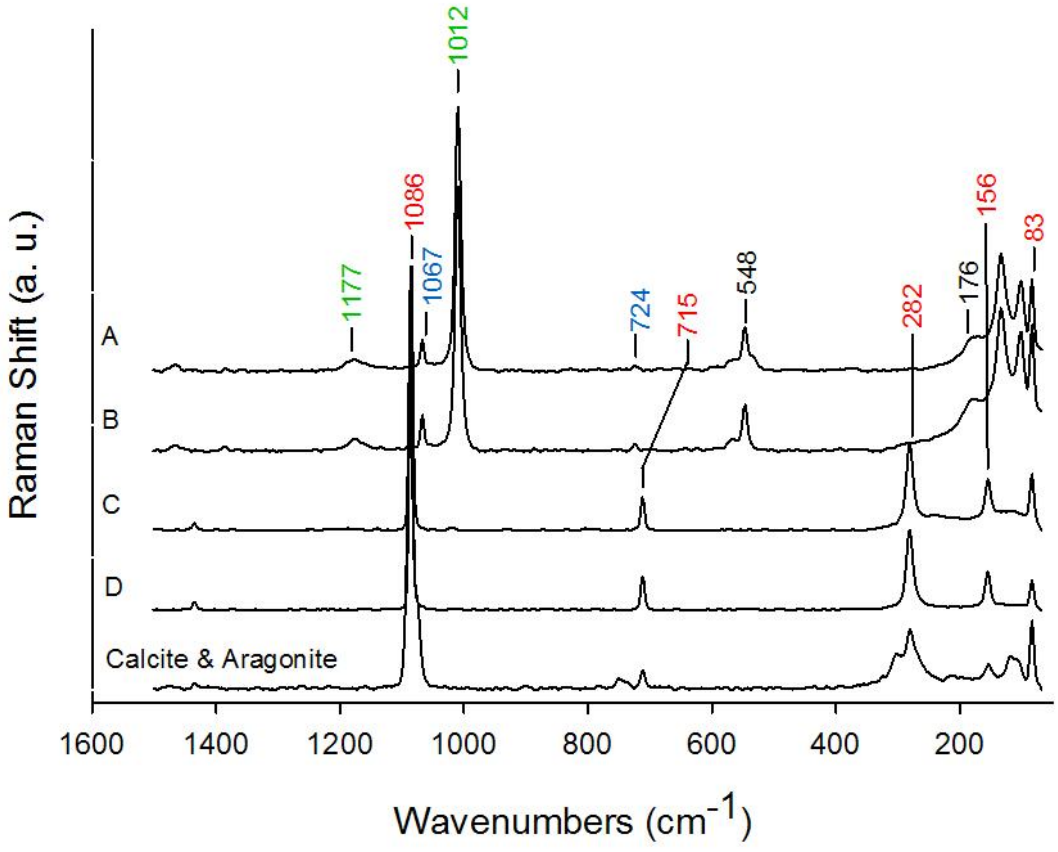

Figure 4.19 Fourier Transform Raman (FT-Raman) spectra of A) urea treated groundwater inoculated with culture 4, B) urea treated groundwater inoculated with culture 3, C) urea treated broth inoculated with culture 4, D) urea treated broth inoculated with culture 3, and E) chemically precipitated calcite and aragonite mixture.

The FTIR-PAS also verified the presence of carbonate in both the broth and groundwater samples (Fig. 4.20). The FTIR-PAS showed no difference between the two inoculants, culture 3 and culture 4. Only the spectra for culture 4 are displayed with the chemical calcite reference (Fig. 4.20). All spectra have peaks at 1467 cm^{-1}, indicating the presence of carbonate as well as small peaks at 1082 cm^{-1} and a split peak at 876 cm^{-1} and 848 cm^{-1}. The split peak at 876 cm^{-1} and 848 cm^{-1} are indicative for the presence of both calcite and aragonite (Table 4.2) (Choi and Kim, 2000; Wang et al., 2006). Both the broth and groundwater spectra have peaks representing the fatty acids at 2876 cm^{-1} and 2930 cm^{-1} and the amide I band at 1653 cm^{-1} and 1646 cm^{-1}. The broth and chemical calcite and aragonite samples both have bands at 2511 cm^{-1} which is likely the O-H stretch in $NaHCO_3$. HCO_3^- is present in the broth media and in the solution for the chemical precipitation of $CaCO_3$.

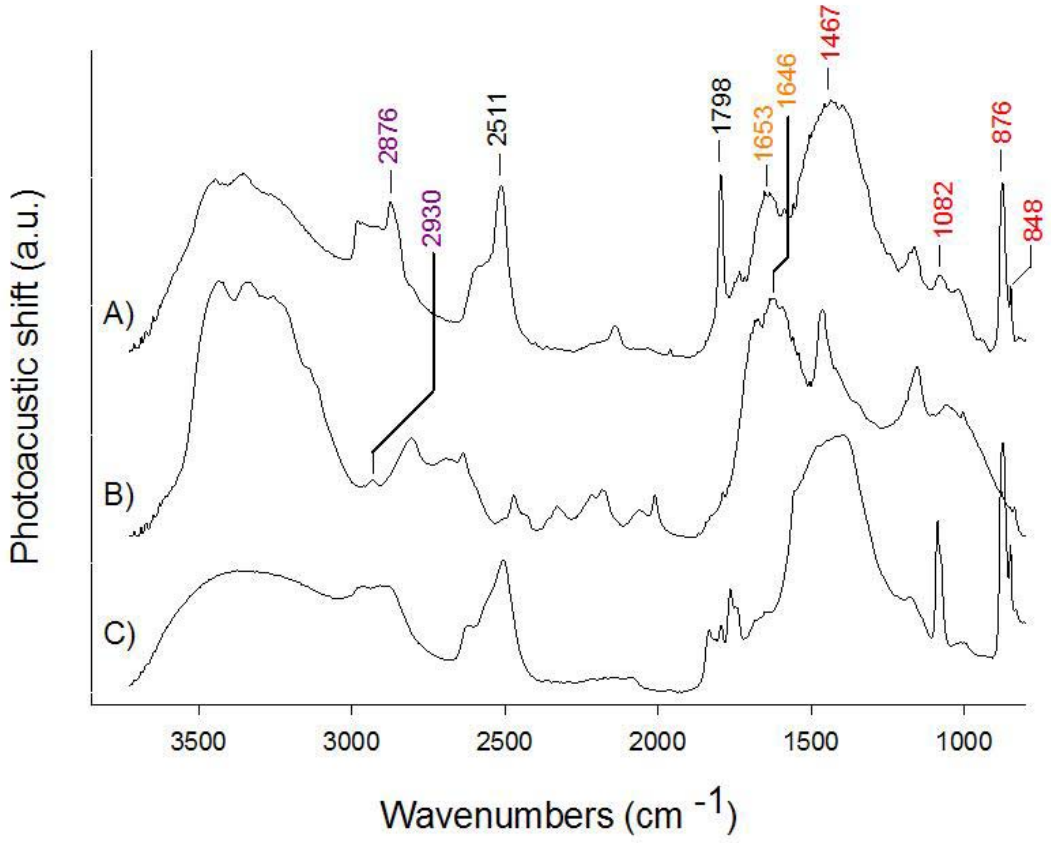

Figure 4.20 Fourier Transform Infrared Photoacustic (FTIR-PAS) spectra of A) urea treated broth inoculated with culture 4, B) urea treated groundwater inoculated with culture 4, and C) chemically precipitated calcite and aragonite mixture standard. The marked peaks represent the presence of calcite and aragonite (876 cm^{-1}, 848 cm^{-1}, 1082 cm^{-1}, and 1467 cm^{-1}), amide I (1646 cm^{-1} and 1653 cm^{-1}), and fatty acids (2876 cm^{-1} and 2930 cm^{-1}).

4.5 Discussion

Inoculation of ureolytic cultures in urea treated broth and groundwater resulted in precipitation of a calcite and aragonite mixture. The denitrification experiments with added nitrate in the broth media decreased free Ca(II) by 56 % and 55 % in the samples inoculated with culture 3 and 4 in the +urea treatment respectively. Free Ca(II) did decrease in the broth samples inoculated with culture 3 and 4 without the addition of nitrate. However, no visible precipitate was formed. The denitrification experiment with groundwater samples +urea treatment decreased free Ca(II) by 11 % and 10 % when inoculated with culture 3 and 4, respectively. Ureolytic $CaCO_3$ precipitation studies by Hammes (2003) resulted in the formation of calcite and some vaterite, which is an unstable polymorph. Hammes used Ca(II) rich wastewater and had a Ca(II) decrease of > 80% in form of ureolytic calcite (Hammes, 2003). Unlike this study, other ureolytic $CaCO_3$ studies by Fujita et al (2004), and Fujita et al (2000) precipitated strictly calcite. Previous studies by Hammes et al (2003), Fujita et al (2000), Fujita et al (2004), Stocks-Fisher et al (1999), and Warren et al (2001) identified the presence of calcite using X-ray diffraction (XRD). Analysis of the precipitate using the different FTIR techniques verified that the $CaCO_3$ precipitated in this study was a mixture of calcite and aragonite and that bacteria was closely associated with the minerals in both the groundwater and broth samples.

Free Ca(II) concentrations decreased in the urea treated groups for both broth and groundwater media. The free Ca(II) only decreased by 11 % and 10 % respectively in culture 3 and 4 inoculated samples in the denitrifier experiment. The broth samples had a greater decrease in Ca(II) in the denitrifier experiment. The free Ca(II) decreased by 56

% and 55 %, respectively, in the inoculated culture 3 and 4 samples of the denitrifier experiment. Although the Ca(II) concentration decreased in the groundwater media, the difference in Ca(II) concentration between the inoculated samples and the control group was not as large as the broth samples. The groundwater samples did not have as much calcite precipitation as the broth samples because of the difference in Ca(II) concentration. Ardkenneth groundwater has a Ca(II) concentration of 32 mg L^{-1} to 46 mg L^{-1}, whereas the broth has a Ca(II) concentration around 1300 mg L^{-1}. Culture 3 had less Ca(II) at the end of 35 d in both experiments in the groundwater samples. Similarly culture 3 decreased Ca(II) more than culture 4 in the first experiment in the broth samples. However, there was no difference between the cultures in the second experiment.

Culture 3 performed better than culture 4 for Ca(II) decrease in both experiments in both media. The ability for culture 3 to decrease Ca(II) independent of nitrate addition may indicate that nitrate does not limit its growth or ability to hydrolyze urea. The findings of this study may indicate that culture 4 is a denitrifier whereas culture 3 is a facultative anaerobe, with the ability to be fermentative. Although Ca(II) concentrations decreased in the first experiment, visible precipitate only formed only when nitrate was added to the broth and groundwater media. Thus the addition of nitrate increased the performance of both cultures in the broth media. However, nitrate did not increase precipitation in the groundwater media. Fujita et al. (2004) precipitated ureolytic calcite using *Bacillus pasteurii*, a known denitrifier, in a synthetic groundwater media. The synthetic groundwater contained 0.04 m*M* of nitrate but nitrate may not have been a limiting factor in their aerobic experiments (Fujita et al., 2004). Hammes (2003) also

precipitated calcite aerobically using ureolytic microorganisms and used urea test agar without nitrate added.

Arsenic concentrations decreased in the urea treated broth samples but not the control samples in both experiments. Culture 3 decreased arsenic concentrations more than culture 4 in the urea treated broth samples in both inoculation experiments. Both inoculants decreased arsenic equally in the denitrifier broth experiment in the urea treated groups, which is similar to the Ca(II) concentration decrease. The denitrifier broth + urea treatment had arsenic loading of 0.91 μg As g^{-1} $CaCO_3$ for culture 3 and 0.85 μg As g^{-1} $CaCO_3$ for Culture 4. There was no difference in arsenic concentration between treatments in the groundwater media. This lack of arsenic decrease could be because low Ca(II) concentrations in the Ardkenneth groundwater resulted in less calcite precipitation. Fujita et al. (2004) removed ^{90}Sr from a synthetic groundwater using ureolytic calcite precipitation. The ^{90}Sr was removed by coprecipitation and replaced the Ca(II) within the calcite crystal matrix (Fujita et al., 2004). This method of contaminant removal was successful, but the synthetic groundwater contained approximately 72 mg L^{-1} of free Ca(II), which is two-fold the Ca(II) concentration of the Ardkenneth groundwater (Fujita et al., 2004). Precipitation of calcite in the broth samples corresponded to the decrease of dissolved arsenic.

The results of this study indicate that inoculation of an isolated calcite precipitating bacterial culture does in fact precipitate calcite in Ardkenneth groundwater. However the precipitation of calcite did not change the arsenic concentration in the Ardkenneth groundwater. Although this technology should not be dismissed, it is not applicable to the Ardkenneth groundwater and other groundwater systems with similar

chemistry. Further studies with ureolytic organisms should be carried out in media with higher free Ca(II) concentrations.

5.0 GENERAL DISCUSSION

The most important finding of the inoculation experiments is that precipitation in the broth media did reduce arsenic concentrations up to 93%. Although the groundwater media did precipitate calcite and aragonite, arsenic concentrations were not reduced as there were very small amounts of precipitate in comparison to the broth samples. The groundwater samples reduced the total free Ca(II) by 11 % and 10 % when inoculated with culture 3 and 4 respectively, whereas the inoculated broth samples reduced total free Ca(II) by 56 % and 55 % for culture 3 and 4 respectively. This greater reduction in the broth could be a result of the increase in urease activity in the presence of higher Ca(II) concentrations as found by Hammes et al. (2003). Hammes et al (2003) found that urease activity increased 10-fold in the presence of high concentrations of free Ca(II), around 30 m*M*, similar to the broth Ca(II) concentration.

Precipitation of a calcite and aragonite mixture was successful when inoculating groundwater with ureolytic cultures. Using a broth media with a high free Ca(II) content formed a visible precipitate identified by IR spectroscopy. The cultures inoculated into the groundwater were isolated from the Ardkenneth groundwater using selective ureolytic agar. The successful precipitation suggests that the introduction of a foreign bacterial population into a groundwater system is not needed for successful calcium carbonate precipitation. Hammes (2003) did not use an inoculant to remove free Ca(II) from Ca(II) rich wastewater, but rather the natural bacterial populations in the raw

wastewater. The batch experiments with the raw wastewater were incubated aerobically for 5 d at 28°C (Hammes, 2003). Hammes (2003) was successful in removing the Ca(II) from the wastewater by ureolytic calcite and associated vaterite precipitation. Fujita et al. (2004) inoculated synthetic groundwater with *Bacillus pasteurii*, and incubated the reaction vessel at 20°C aerobically for 8 h. The reaction experiments in the synthetic groundwater were successful in sequestering ^{90}Sr in ureolytic calcite (Fujita et al., 2004)

Two cultures were used as inoculants in this study, based on both aerobic and anaerobic selection experiments detailed in Chapter 4 (section 4.2.1). Both inoculants had some ureolytic activity in the test broth media in both the fermentative and denitrification experiments. Culture 3 decreased free Ca(II) concentrations in the broth independent of nitrate addition and lowered free Ca(II) by 43 % in the first fermentative experiment. Inoculation of culture 4 in the same experiment resulted in a loss of only 35 % free Ca(II), which is less than culture 3. This finding suggests that ureolytic activity could be affected by the redox conditions of the system and whether an adequate source of NO_3 is available. Although no visible precipitate was formed in the fermentation experiment, it is possible to precipitate $CaCO_3$ by fermentation if there is adequate concentrations of free Ca(II) and the presence of culture 3. Unlike this study, previous ureolytic $CaCO_3$ studies were also conducted aerobically using either a broth or synthetic groundwater media (Fujita et al., 2000; Fujita et al., 2004; Stocks-Fischer et al., 1999; Warren et al., 2001). This study was successful in $CaCO_3$ precipitation in an anaerobic environment, which may be important for deep aquifer systems.

Precipitation of the calcite/aragonite mixture in the broth media reduced arsenic concentrations significantly. The reduction of arsenic in the broth samples indicates that the ureolytic precipitation of calcium carbonate will sequester and remove arsenic from

the system. Arsenic was reduced in the broth samples only because of the high free Ca(II) concentrations, resulting in a greater yield of precipitate at the end of the experiment. The denitrification experiment in the broth media had arsenic loadings of 0.91 μg As g^{-1} $CaCO_3$ for culture 3 and 0.85 μg As g^{-1} $CaCO_3$ for culture 4. Fujita et al. (2004) had a Sr:Ca ratio of 0.044 when the synthetic groundwater was inoculated with *B. pasteurii* and urea was added to the system. The Sr:Ca ratios were determined using inductively coupled plasma with atomic emission spectrophotometric detection and turned out to be higher for the ureolytic calcite compared to the chemically precipitated calcite using ammonium carbonate treatments (Fujita et al., 2004).

Inoculation of ureolytic cultures isolated from the Ardkenneth groundwater resulted in the precipitation of a calcite/aragonite mixture. Splitting of the bands at 876 cm^{-1} and 848 cm^{-1} indicates that a mixture of calcite and aragonite was formed in all urea treatments inoculated with the two ureolytic cultures in the inoculation experiment with added nitrate (Choi and Kim, 2000; Wang et al., 2006). Inoculation of the two selected cultures into the broth media also resulted in the precipitation of a calcite/aragonite mix. Previous ureolytic precipitation studies precipitated calcite, with the exception of Hammes et al (2003). Hammes (2003) had calcite with some associated vaterite, identified by XRD (Fujita et al., 2000; Fujita et al., 2004; Hammes et al., 2003; Stocks-Fischer et al., 1999; Warren et al., 2001). This study used several different IR techniques for $CaCO_3$ identification, IR microscopy was used for identification of CO_3 bands in the region of 1459 cm^{-1} and 1463 cm^{-1}. This is indicative of a $CaCO_3$ but does not distinguish between the three polymorphs. Also, a calcite/aragonite mixture standard did not result in a usable data as the small particle size affected the spectrum. Further work with FT-Raman spectroscopy compared the precipitate samples with the calcite

aragonite standard. The FT-Raman confirmed the presence of calcite in the precipitate. Spectroscopy studies using FTIR-PAS resulted in confirmation of the calcite aragonite mixture within the precipitate samples. The different spectroscopy techniques also indicated a close relationship in the precipitate and bacterial cultures as some cell structures were present in the spectra.

This study identified calcite and aragonite precipitates only, no other precipitates were formed in either the groundwater or broth media by both visual and spectroscopic observations. Arsenic remediation is often associated with oxides. Studies by Sun and Doner (1996) have reduced intensity FTIR bands for adsorbed As(III) and As(V) on the goethite surface at 2701 cm^{-1}, 2681 cm^{-1}, and 2584 cm^{-1}. In the present study the bands indicative for goethite were not present in either the groundwater or broth media. The broth media had no Fe in solution and the Ardkenneth groundwater contained minimal Fe (0.6 to 1.45 mg L^{-1}). Subsequently, FT-Raman bands characteristic for Al oxides at 502 cm^{-1} and 668 cm^{-1} were not present in this study for either the groundwater or broth media (Goldberg and Johnston, 2001). The lack of competing sorption sites for arsenic in this study suggests that the precipitation of calcite and aragonite reduced the arsenic concentrations in the broth media.

Potential for contaminant remediation using ureolytic calcite precipitation still exists, but more research is warranted. This study determined that the precipitation of calcite in the Ardkenneth groundwater did not reduce arsenic concentrations. Arsenic concentrations were not reduced simply because insufficient calcite was precipitated in the system, which was limited by the Ca(II) concentration of the groundwater. When the concentration of free Ca(II) is conducive to high yields of $CaCO_3$, ureolytic precipitation

of $CaCO_3$ reduced arsenic concentrations by 88%, a promising step in arsenic remediation.

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APPENDIX A

A.1 Calcium Ion Selective Electrodes

Ion selective electrodes (ISE) are widely used in analytical chemistry as they offer relatively inexpensive analysis independent of sample turbidity or colour. Electrodes provide analysis with high-speed and with increasing accuracy as design and sensor development improve (Bailey, 1980). The most widely known ISE is the pH electrode, which determines the activity of H^+ ions in a solution or a solid.

ISE's assess ion activity in solution based on membrane junction potential. The activity of the ions can then be related to the concentration of ions in solution (Bailey, 1980). The potentiometric measurement is based on the potential difference across the membrane. As ions move across the membrane they create a build up of positive charge on the inside of the membrane. The positive charge created establishes an electrical potential difference across the membrane (Bailey, 1980). Potentiometry is expressed by the Nernst equation which deals with cell potentials in addition to energy involved in chemical reactions (Rayner-Canham, 2000a; Solsky, 1990a). The general Nernst equation correlates the Gibbs free energy (ΔG) and the electromotive force (EMF) of a galvanic cell or chemical system (Rayner-Canham, 2000a).

$$aA + bB = cC + dD$$

$$\text{So: } Q = \frac{[C]^c + [D]^d}{[A]^a + [B]^b}$$

$$\text{If: } \Delta G = \Delta G^\circ + RT \ln Q$$

$$\text{and } \Delta G = -nF\Delta E$$

$$\text{Then: } \Delta E = \Delta E° - \frac{RT \ln[C]^c + [D]^d}{nF[A]^a + [B]^b}$$

Eq. A.1

The charge buildup on the membrane of the ISE will eventually result in the loss of charge of the silver ions of the internal reference solution. This loss of charge will cause electron deposition onto the wire in the inside of the ISE, thus drawing electrons through the external wiring of the meter and from the reference solution (Bailey, 1980). This transfer of electrons compensates for the lack of positive charge resulting from the loss of ions. At equilibrium, this flow of electrons will stop, though a residual voltage difference will remain. The measured potential, in millivolts (mV), is the sum of all of these voltage potentials.

The Ca^{2+} ISE is an electrode specific to analysis of the Ca(II) as the primary ion. Similar to most ISE, a calcium electrode has a membrane composed of polymer-matrix substances, specifically polyvinyl chloride (PVC) (Solsky, 1990b). The PVC membrane of a calcium selective electrode contains an organic molecule or ionophore, such as $C_{38}H_{72}N_2O_8$ or $C_{29}H_{37}N_3O_6$, which is specific for binding calcium. The Ca^{2+} ISE functions the same as all ISE in measuring the potential difference in mV across the PVC membrane.

Common sources of error when using an ISE are temperature, ionic strength, pH, competing ions, and electrode drift (Bailey, 1980). Temperature is very important as a single degree change in temperature can result in error greater than 4% (Bailey, 1980). Change in temperature affects the slope of the electrode response, but also affects the reference solution. Heat transfer across the membrane can also effect electrode readings

(Bailey, 1980). ISE's do not automatically correct for temperature, so it is important that both standards and samples are kept at the same temperature (Bailey, 1980). Potential error because of ionic strength can be avoided with an ionic strength adjuster (ISA) or by having the ionic strength of the standards similar to the samples. This reduces the potential for error (Bailey, 1980). Hydrogen ion (H^+) interference can be avoided by adjusting the pH of the sample solution (Bailey, 1980). At lower pH values (< 4) the calcium ISE will measure hydrogen ion activity, not Ca(II) (Bailey, 1980). Ions with similar chemical characteristics and valency will potentially interfere with ISE cation measurements (Bailey, 1980). In the case of a calcium ISE, magnesium [Mg(II)] and H^+ are often competing ions depending on the concentration of Mg(II) and pH of the sample solution (Bailey, 1980). To correct for competing ions buffers of specific concentrations can be used to mask Mg(II) ions, and adjusting sample pH will correct for H^+ interference (Bailey, 1980). Electrode drift is often an issue when using the Ca(II) ISE. To avoid error from electrode drift frequent calibration of the ISE is necessary, as drift can be as high as 2-5 mV per day (Bailey, 1980).

A.2 Ca^{2+} Electrode verification with Atomic Adsorption

Calcium electrode measurements were verified using atomic absorption (AA) on 12 random samples. Some of the samples are repeated, to verify AA precision as well. the AA calibrated with an R^2= 0.999 using the same Ca(II) calibration solutions that were used for the Ca(II) electrode. Table A.1 shows the values in mg L^{-1} determined by both the Ca(II) electrode and the AA using the Ca(II) lamp. The experiment corresponds to the bioreactor experiment that the samples were obtained from, and the sample name refers to the urea treated (U) or the control (C) sample with the number representing the replicate. A 2 sample t-test with a 95% confidence interval determined that the Ca(II) concentrations for the AA were not significantly different from the Ca(II) concentrations from the electrode (p=0.48; SD=4.33).

Table A.1 Values in mg L^{-1} for Ca(II) concentration obtained from 12 randomly selected samples from bioreactor experiments 2 and 4 for the Ca(II) electrode and the AA. There was no significant difference between the AA and Ca(II) electrode confirmed by a 2 sample t-test.

Bioreactor Experiment (#)	Sample Name	AA (mg L^{-1})	Electrode (mg L^{-1})
4	U-1	15	17
2	U-1	24	24
2	U-2	20	20
2	U-3	13	15
2	U-1	21	21
2	U-1	21	21
4	U-1	15	17
4	C-1	18	18
2	U-3	17	18
2	U-3	13	15
4	U-3	23	27
4	U-3	26	27

APPENDIX B

6.1 B.1 Wheatman Celstir Bioreactor Flask Schematics

A schematic diagram for the first two open flow-through bioreactor experiments (Fig. B.1), showing how the bioreactors were set up. The flow through bioreactors had groundwater flow through in one direction at the end of the 5 day cycle after groundwater was sampled for OC, and arsenic analysis. The groundwater would flow in/out of the arms of the bioreactor near the top of the flask, leaving any bacteria in the system that were causing nucleation of $CaCO_3$ precipitation, as they would gravitate towards the bottom of the bioreactor.

The third and fourth bioreactor experiments were non flow-through experiments, meaning that there was no one directional flow of groundwater through the bioreactor flasks (Fig. B.2). The bioreactors were closed, and groundwater was sampled on day 0 of the experiment for initial analysis of OC and arsenic, and the final day of the experiment for final analysis of OC and arsenic.

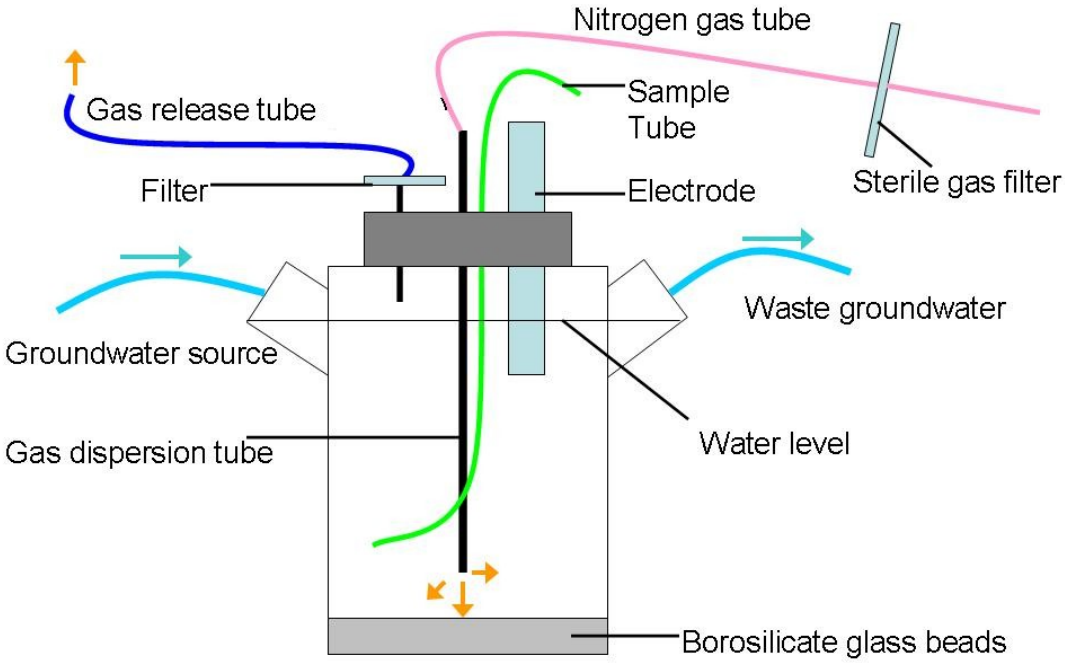

Figure B.1 Schematic representation of a single 250 mL Wheatman Celstir water jacketed bioreactor flask in the flow-through bioreactor experiment. The groundwater would flow through the bioreactor in one direction at the end of every 5 day cycle. The unidirectional flow of the groundwater would replicate aquifer conditions as well as serve to rid the system of any bacterial cells not nucleating $CaCO_3$ crystal growth. The Eh was monitored with platinum redox electrodes and the system would be sparged with N_2 gas when needed for 5 minutes at a time during the second bioreactor experiment.

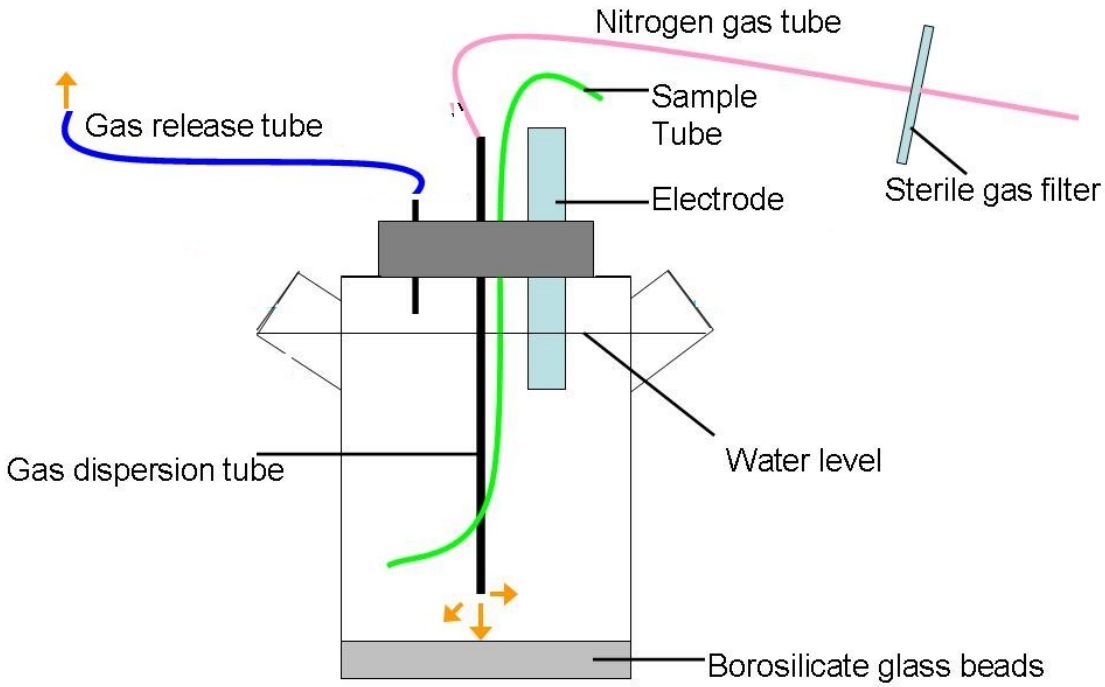

Figure B.2 Schematic representation of a single 250 mL Wheatman Celstir water jacketed bioreactor flask in the non flow-through bioreactor experiment. The system would remain closed through the duration of the experiment, with groundwater sampled at day 0 and the final day of the experiment. The Eh was monitored with platinum redox electrodes and the system would be sparged with N_2 gas when needed for 5 minutes at a time.